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<b>(54) Title:</b> METHOD OF INHIBITING TRANSFORMATION, GROWTH AND METASTASIS OF CELLS IN WHICH PURINE METABOLIC ENZYME ACTIVITY IS ELEVATED  <b>(57) Abstract</b> <p>A method of inhibiting growth, transformation, and/or metastasis of mammalian cells, particularly epithelial cells, in which activity of at least one enzyme, which participates in purine metabolism or regulation of nucleotide levels or the relative ratios of their phosphorylated states, is elevated. In particular, a method of inhibiting transformation, growth and/or metastasis of mammalian cells in which a DNA tumor virus, a DNA tumor virus factor or other factor which has an equivalent effect on cells has acted.</p>		

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METHOD OF INHIBITING TRANSFORMATION, GROWTH AND  
METASTASIS OF CELLS IN WHICH PURINE METABOLIC ENZYME  
ACTIVITY IS ELEVATED

Description

Background

Transformation, or malignant transformation, of cells results in changes in their growth characteristics and can cause them to form tumors in animals into whom they are introduced. For example, transformation of adherent cells can be associated with alterations such as changes in growth control, cell morphology, membrane characteristics, protein secretion and gene expression. Although transformation can occur spontaneously, it can be caused by a chemical or irradiation or may result from infection by a tumor virus. Little is known about the underlying molecular events. One type of RNA viruses (the retroviruses) and many different types of DNA viruses can act to transform cells and collectively are referred to as tumor viruses. In the case of tumor viruses, it is clear that the virus does not itself carry all of the genes necessary to produce the phenotypic changes characteristic of infected cells. Tumor viruses may act through a gene or genes in their genome (oncogenes) which, in some way, influence or induce target cell genes. The induced target cell genes, in turn, act to carry out the changes observed in transformed cells. There are at least three major

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classes of transforming DNA viruses: adenoviruses, which have two groups of oncogenes, E1A and E1B, which act together to produce transformation; papovaviruses, which synthesize proteins, called T antigens, which may work together to transform cells; and herpes viruses, for which no oncogene has been identified as yet.

Although considerable effort has been expended in identifying transforming genes or oncogenes and, in some cases, has also resulted in identification of their protein products, very little is known about the cellular mechanisms affected in the transformation process. There is a consensus that these oncogenes perturb cell growth by modifying the expression or activity of key growth related genes. It would be very helpful to have a better understanding of how transformation occurs, particularly if the biochemical pathways affected can be identified. Such knowledge would make it possible to design compounds which can interfere with or counter the effects of the transforming signals and, thus, are useful in preventing transformation or minimizing the extent to which it occurs, once begun, and, thus, to reduce effects on individuals in whom it occurs.

#### Disclosure of the Invention

The present invention relates to a method of inhibiting growth, transformation and/or metastasis of mammalian cells in which activity of at least one purine metabolic enzyme is elevated (i.e., is



greater than that in the corresponding untransformed or normal cell). As used herein, the term purine metabolic enzyme includes any enzyme which participates in purine metabolism or in regulation of nucleotide levels or the relative ratios of the phosphorylated states (e.g., ATP/ADP), or both. In particular, the present invention relates to a method of inhibiting (preventing, reducing or reversing) transformation of mammalian cells by a DNA tumor virus, a DNA tumor virus factor (or product) or other factor (e.g., a cellular factor) which has an equivalent effect on cells as that of the DNA tumor virus or DNA tumor virus factor (i.e., causes, either directly or indirectly, an increase in purine metabolic enzyme activity). The present invention also relates to a method of inhibiting growth and/or metastasis of mammalian cells in which such a virus, virus factor or other factor has acted. The method is carried out by inhibiting (or interfering with) the increase in purine metabolic enzyme activity caused by the DNA tumor virus or factor, by contacting cells in which the level is increased with a drug capable of causing the desired effect (i.e., inhibition of purine metabolic enzyme activity). This is carried out, for example, by interfering with the ability of the virus or the factor to increase expression of a gene(s) which encodes a host cell purine metabolic enzyme(s) or by counteracting the increase in purine metabolic enzyme activity, directly or indirectly. In carrying out the present method, cells in which the

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purine metabolic enzyme level is elevated are contacted with the drug (or drugs) under appropriate conditions: that is, conditions appropriate for the drug to pass into the cell (in those embodiments in which an intracellular effect is necessary) or under conditions appropriate for the drug to remain at or within cell membranes (in those embodiments in which an effect at or within the cell membrane is necessary). The present invention further relates to compounds or drugs useful in reducing the effects on cells of a DNA tumor virus, DNA tumor virus oncogenic product, or other factor.

Drugs useful in the present method can have the desired effect on cell transformation, growth or metastasis by acting to combat an effect of the viral product or other factor or to interfere with action of the viral product in one or more of the following ways: by inhibiting the purine metabolic enzyme whose activity in the cell is elevated; by decreasing the message level; by interfering with the transcription factors which induce expression of the host cell purine metabolic enzyme(s); by inhibiting the interaction of the viral product or other factor with a host cell gene(s) encoding a purine metabolic enzyme(s); and by inhibiting interaction of the viral product or factor with a host cell gene product(s) that regulates or that is recruited to regulate the activity or expression of the purine metabolic enzyme(s).

Drugs useful in the present method can be existing drugs, analogues of existing drugs or drugs

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designed specifically for the purpose of reducing purine metabolic enzyme activity, such as by inhibiting the DNA tumor virus or its activity. The present method is particularly useful in blocking the ability of a papillomavirus oncogenic product (e.g., an E7 product) to increase the expression of the host cell creatine kinase gene, or other host cell gene(s) which participates in purine metabolism (e.g., in cervical epithelial cells). Thus, it is useful in inhibiting transformation of such cells.

Other tumors which are characterized by elevated activity of a purine metabolic enzyme(s) (i.e., greater activity than in the corresponding untransformed or normal cell) and which are associated, directly or indirectly, with the presence of the DNA tumor virus, tumor virus factor or other factor can be treated in a similar fashion. For example, tumors that result from cellular mutations that mimic the effects of infection by a DNA tumor virus can be treated in a similar manner. For instance, loss of anti-oncogene products Rb, DCC or p53 may mimic infection by a DNA tumor virus, leading ultimately to elevated activity of a purine metabolic enzyme(s). These tumors are likely candidates for treatment by the present method.

Through use of the present method, it is possible to prevent, reduce or reverse transformation of mammalian cells by DNA tumor viruses, DNA tumor virus factors or other factors that have an equivalent effect on cells and to

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prevent, reduce or reverse growth and/or metastasis of transformed cells in which such a virus, virus factor, or other factor has acted. Thus, through use of the present method, it is possible to prevent tumor formation, reduce the extent to which tumor formation occurs, or to reverse the progress (e.g., growth or metastasis) of a primary or metastatic tumor.

#### Brief Description of the Drawings

Figure 1 shows colocalization of the E7 and E1a target sequences in the E2 promoter attached to the CAT gene.

Figure 2 shows the structural similarity between adenovirus E1a and papillomavirus HPV-16 E7 transforming proteins.

Figure 3 shows the sequence relationship between the adenovirus E2E promoter and that of the B isozyme of creatine kinase, with lines connecting identical nucleotides.

Figure 4 shows that the 12S product of E1a (which encodes the transforming protein) lacking the transactivation function activates expression of creatine kinase B (CKB).

Figure 5 shows that point mutations in domain 2 of E1a which disrupt transformation are also impaired for E1a-induced expression of CKB.

Figure 6 shows that amino-terminal deletions of E1a which disrupt transformation also result in an inability to induce expression of CKB.

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Figure 7 shows part of the sequence of CKB gene, from which antisense oligonucleotides useful to block CKB translation can be determined.

Figure 8 is a graph illustrating the dose-related response of the DU 145 prostate carcinoma cell line to varying concentrations and durations of treatment with a homocyclocreatine preparation. The effects of 1.0 mM, 2.0 mM, 5.0 mM and 10.0 mM homocyclocreatine preparation on total incorporation of  $^3\text{H}$ -thymidine are plotted as a percent of the untreated control for days 1 through 5, versus the concentration of homocyclocreatine preparation.

Figure 9 is a bar graph illustrating the inhibition of growth and the dose-related response of five colon carcinoma cell lines (SW1116, SW48, SW620, CACO2, and WIDR) to varying concentrations of cyclocreatine (0 mM, 3.5 mM, 7.0 mM, 14.0 mM, 28.0 mM, 42.0 mM, 56.0 mM and 70.0 mM) after 7 days of treatment. The height of each bar represents the counts per minute (cpm) of  $^3\text{H}$ -thymidine incorporated at each drug particular concentration.

Figure 10 is a series of bar graphs illustrating the effect of 4 mg/ml drug on growth (as assayed by incorporation of  $^3\text{H}$ -thymidine) of various cell lines as a percent of the untreated control. The cell lines include kidney (Vero, 293), embryonal (BALB/c3T3 (BALB)) lung (MRC-5, NCI-H69 (H69)), cervical (C-33A, HeLa, SiHa, CaSki, and ME-180), prostate (LNCaP.FGC (LNCAP), DU 145, and PC-3) and colon (SW1116, SW403, and SW48) cell

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lines. Figure 10A shows the effect of a homocyclocreatine preparation on growth of the cell lines; Figure 10B, shows the effect of a 1-carboxymethyl-2-imino-hexahydropyrimidine preparation on growth of the cell lines; Figure 10C, shows the effect of cyclocreatine on growth of the cell lines; Figure 10D, shows the effect of guanidinoacetate on growth of the cell lines.

Figure 11 is a bar graph illustrating one of the observed trends of the antiproliferative activity of homocyclocreatine and 1-carboxymethyl-2-imino-hexahydropyrimidine on the one hand, in comparison to that of cyclocreatine. In this drawing the height of the bar represents the incorporation of  $^3\text{H}$ -thymidine as a percent of the untreated control for cervical carcinoma cell lines C-33A, HeLa, SiHa, CaSki and ME-180 on treatment with 4 mg/ml homocyclocreatine (HcCr), 1-carboxymethyl-2-imino-hexahydropyrimidine (6CCr) or cyclocreatine (CCr).

Figure 12 is a graph illustrating the anti-proliferative activity of cyclocreatine in a human tumor xenograft model. The average volume ( $\text{mm}^3$ ) of SW48-derived colon carcinoma tumors implanted in nude mice fed 0.0 %, 0.5%, or 1.0% dietary cyclocreatine is plotted against time (days post-implantation).

Figure 13 is a graph illustrating the anti-proliferative activity of cyclocreatine in a second human tumor xenograft model. The average change in volume ( $\text{mm}^3$ ) of ME-180-derived cervical

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carcinoma tumors implanted subcutaneously in nude mice fed 0.0 %, 0.5%, 0.75% or 1.0 % dietary cyclocreatine is plotted against time (days post-implantation). Drug regimens were not initiated until 24 days after tumor cell implantation.

#### Detailed Description of the Invention

Oncogenic products of DNA tumor viruses, particularly those DNA tumor viruses associated with transformation or tumors of epithelial cells, have been shown to have certain functional, structural and sequence similarities which make it reasonable to expect that they have a common or similar effect on host cells which results in induction of cellular transformation. In addition, activity of an enzyme which participates in purine metabolism or in regulation of nucleotide levels or the relative ratios of the phosphorylated states (i.e., a purine metabolic enzyme) is known to be elevated (increased) in transformed mammalian cells. In particular, it is known that the activity of brain creatine kinase (CKB or CKBB), which is a purine metabolic enzyme, is characteristically elevated in transformed cells and tumors in which a DNA tumor virus or DNA tumor virus factor is present or on which such a virus or virus factor has acted, resulting in transformation or tumor formation. CKB is characteristically elevated in many types of tumors and is a reliable marker for at least one tumor type (small cell lung carcinoma). CKB is

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induced by at least one DNA tumor virus (i.e., adenovirus) and CKB induction correlates with the ability of the oncogenic region of the virus to transform cells.

In particular, it is known that the oncogenic products of two DNA tumor viruses, the E7 protein of human papillomavirus (HPV) 16 and the adenovirus E1a protein, have many similar functions in cells and are able to increase host gene expression. As described in detail herein, these similarities and the ability shown by these DNA viruses to induce cellular transformation appear to be linked to their ability to increase host cell gene expression, particularly their ability to increase expression of purine metabolic enzymes.

The present invention relates to a method of inhibiting growth, transformation, and/or metastasis of mammalian cells in which activity of one or more purine metabolic enzymes is elevated. This is carried out by inhibiting (reducing or eliminating) the ability of: (1) a DNA tumor virus, (2) a factor produced by or characteristically associated with a DNA tumor virus (a DNA tumor virus factor) or (3) other factor (e.g., a cellular factor) which has an effect corresponding to that of the DNA tumor virus or virus factor (a DNA tumor virus factor equivalent), to induce cellular transformation or to maintain a transformed phenotype and to cause an elevation of purine metabolic enzyme activity. The method is effected by interfering with the ability

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of the virus or factor to increase expression of such purine metabolic enzymes or by countering the increase in purine metabolic enzyme activity. Compounds or drugs useful in the present method are also the subject of the present invention.

In particular, through use of the present method, it is possible to inhibit the ability of a DNA tumor virus, a DNA tumor virus factor, or other factor which has an equivalent effect on cells, to increase the expression of host cell enzymes, such as creatine kinase, adenylate kinase, adenylate cyclase and adenosine kinase, which participate in purine metabolism or which interact with these host cell enzymes. It is also possible by the use of inhibitors of these enzymes to counterbalance the induction effect caused by the virus. Thus, it is possible to inhibit the ability of the DNA tumor virus, DNA tumor virus factor, or other factor to induce cellular transformation or to maintain the transformed state. As a result, cellular transformation, maintenance of transformation, growth and/or metastasis is inhibited (i.e., it is prevented or occurs to a lesser extent than would be the case if the present method were not carried out or is reversed, wholly or in part, once a cell has been transformed).

The present method of inhibiting transformation of mammalian cells in which purine metabolic enzyme activity is elevated (i.e., greater than purine

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metabolic enzyme activity in untransformed cells) can have its effect by several different mechanisms. For example, the ability of a DNA tumor virus, DNA tumor virus factor or other factor to induce transformation can be interfered with by:

- 1) inhibiting the purine metabolic enzyme(s) whose activity in transformed cells is elevated as a result of the effect of the virus or factor. This can be carried out, for example, by administering a compound or drug which reduces the activity of the purine metabolic enzyme(s) or reduces the association of two or more purine metabolic enzymes;

- 2) decreasing the message level/preventing translation of the mRNA. This can be carried out by administering antisense constructs which are oligonucleotides selected to bind to a region of the cellular purine metabolic enzyme mRNA necessary for translation. For CKB, for example, one such region has been defined and is that region at the 3' end, shown in Figure 7;

- 3) interfering with transcription factors. This can be carried out by modifying the activity or association of unique factors with their specific promoters. For example, drugs can be designed to interfere with (prevent or modify) the association of transcription factors with genes encoding purine metabolic enzymes, in order to control the extent of initiation and the amount of enzyme produced;

- 4) by inhibiting interaction of viral products with host cell genes encoding the purine metabolic enzymes. This can be carried out by identifying the

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target and preventing the association with and/or activation of the promoter. The unique TTAA element on the CKB promoter, which is shared with the adenovirus E2E promoter, might be an excellent candidate; and

5) by inhibiting interaction of viral product(s) with host cell product(s), that regulate or are recruited to regulate the activity or expression of purine metabolic enzymes. This can be carried out by means of compounds designed to bind to the viral products and to inhibit binding of the host cell product with the viral product.

Because it is known that the activity of purine metabolic enzymes, such as creatine kinase, is elevated in mammalian cells transformed by a DNA tumor virus or virus factor, and because there are enzymes which work in conjunction with creatine kinase, such as adenosine kinase, adenylate kinase and adenylate cyclase, such enzymes might be useful as markers for identifying transformed cells. For example, the occurrence (presence/absence) or quantity of one or more of these enzymes can be determined, using known enzyme assays or specific probes from the isolated genes. The occurrence or quantity of one or more of the enzymes can be an indicator or marker useful for detecting transformation or viral infection of cells. The information can be used for diagnostic purposes or for monitoring treatment in an individual in whom diagnosis has been made.

The present method of inhibiting cellular transformation, growth and/or metastasis can be carried out using existing drugs, analogues of such drugs, or drugs specifically designed for the purpose. Drugs can be administered to produce inhibition of cellular transformation by direct interaction with the virus (e.g., by inhibiting production of viral DNA or mRNA expression of the encoded protein); by blocking the domain or sequence of the virus which is responsible for inducing expression of a cellular gene involved in cell growth perturbing functions; by blocking the activity of a factor, produced by or characteristic of the DNA tumor virus, which is the inducer of such a cellular gene; or by acting directly upon host cell products which interact with a viral product to cause transformation. Drugs may act at the enzyme level as well, so that the activity of the purine metabolic enzyme(s), which is elevated in the cell is reduced. Drug analogues and drugs designed for the specific purpose intended are also the subject of the present invention.

The following is a description of DNA tumor viruses able to induce cellular transformation, which can be inhibited by the present method; the basis upon which this inhibition is effected; the use of the present method in inhibiting cellular growth and/or transformation and compositions useful in the present method. It is important to note that in each of the instances described, in which cellular transformation occurs, the activity of host

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cell creatine kinase is increased (is greater than the activity which occurs in the absence of transformation). Therefore, it is reasonable to expect that the present method can be used in the treatment of other tumors in which the activity of creatine kinase is increased significantly above activity in normal cells. Similarly, it is reasonable to expect that the present method can be used in inhibiting transformation of cells in which activity of other purine metabolic enzymes, particularly those which associate functionally with CKB, is increased. It is also possible to inhibit growth and/or metastasis of transformed cells (reduce the extent to which it occurs or prevent its occurrence) using the method and drugs described.

#### DNA Tumor Viruses

##### A) Human Papillomaviruses and Association with Human Anogenital Cancers

As discussed by Phelps et al., recent studies have established a strong association between certain human papillomaviruses (HPVs) and some types of human anogenital cancers (Phelps, W.C. et al., Current Topics in Microbiological and Immunology, 144:153-166, Springer-Verlag, Berlin Heidelberg, (1989)). More than a dozen different HPV types have been isolated from epithelial tumors of the genital region. Precancerous lesions, such as moderate to severe cervical dysplasia and carcinoma in situ, as well as invasive cervical carcinoma, have been



associated with HPV types 16, 18, 31 and 33 (Zur Hausen, H. and A. Schneider, 1987, In: Howley, P.M., Salzman, N., Eds. The Papovaviridae: The Papillomaviruses, Plenum, NY, pp. 24-263). Of the HPVs which have been associated with anogenital malignancies, HPV-16 has been detected most frequently (greater than 60%) in biopsies from cervical carcinoma. RNA analyses for cervical carcinoma tissues and derived cell lines have revealed that the E6 and E7 ORFs are generally expressed, suggesting that their gene products may be necessary for the maintenance of the malignant phenotype. (Schneider-Gadicke, A. and E. Schwarz, EMBO J., 5: 2285-2292 (1986); Smotkin, D. and F.O. Wettstein Proc. Natl. Acad. Sci. USA, 83:4680-4684 (1986); Baker, C.C. et al., J. Virol, 61:962-971 (1987); Takebe, N. et al., Biochem. Biophys. Res. Commun., 143:837-844 (1987)). Morphological transformation of established rodent cells has been described for HPV-16 (Yasumoto, B. et al., J. Virol, 57:572-577 (1986); Tsunokawa, Y. et al., Proc. Natl. Acad. Sci. USA, 83:220-2203 (1986); Kanda, T. et al., Jpn. J. Cancer Res., 78:103-108 (1987)). This transforming activity has been localized to the E6/E7 region (Bedell, M.A. et al., J. Virol., 61:3635-3640 (1987); Kanda, T. et al., ibid, 62:610-613 (1987)). In addition, the HPV-16 E6/E7 region has been shown to encode a function capable of cooperating with an activated ras oncogene in the transformation of rat embryo fibroblasts

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(Matlaszewski, G. et al., EMBO J., 6:1741-1746 (1987)).

The immortalization function of HPV 16 has been mapped to the E7 ORF by Phelps and co-workers. (Phelps, W.C. et al., Current Topics in Microbiological and Immunology, 144:153-166, Springer-Verlag, Berlin Heidelberg, (1989)). These researchers also found the E7 product of HPV-16 to have activities similar to those of the oncogenic product of another virus, the E1a protein of adenovirus.

#### B) Adenoviruses

Adenoviruses are a class of viruses which cause upper respiratory infections in many animals. They have been isolated from a wide range of species, and at least 31 different serotypes of human adenoviruses have been characterized (Luria et al., General Virology, pp. 360, 3rd Ed., Wiley & Sons, New York (1978)). The basic molecular biology of these viruses is very similar. The early adenovirus gene products are oncogenes that have been shown to be able to immortalize primary cells in culture. In collaboration with a second oncogene, such as the H-ras gene, they can transform cells in vitro.

The proteins encoded by the E1a region of the adenovirus genome are necessary for efficient viral replication and for viral transformation of cells in culture. The E1a proteins contain three distinct domains that are strongly conserved among adenovirus subgroups and species (van Ormond, H. et al., Gene

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12:63 (1980); Kimelman, D. et al., J. Virol. 53:99 (1985)). The vast majority of domain 3 is unique to the 289 amino acid protein and is important for trans-activation of early viral promoters (Berk, A.J., Ann. Rev. Genet. 20:45 (1986)). Domains 1 and 2 are required for transcriptional repression, transformation, and induction of DNA synthesis, but not for activation of transcription (Lillie, J.W. et al., Cell 46:1043 (1986); Lillie, J.W. et al., Cell, 50:1091 (1987)).

C) Similarities Between Human Papillomavirus E7 Oncogenic Product and Adenovirus E1a Oncogenic Product

In summary, the papillomavirus oncogenic product and the adenovirus oncogenic product described above have been shown to have the following similarities:

- 1) Phelps et al. have shown that E7 has transcriptional transactivation properties analogous to those of adenovirus E1a. That is, E7 can affect heterologous promoters, including the adenovirus E2 promoter (Phelps, W.C. et al., In: Current Topics in Microbiology and Immunology: Transforming Proteins of DNA Tumor Viruses, Knippers, R. and Levine, A.J., Eds., Springer-Verlag, Berlin, Heidelberg, pp. 153-166 (1989)). The adenovirus E2 promoter sequences required for E1a stimulation and

HPV-16 E7 activation have been shown to be coincident.

Figure 1 is taken from Phelps et al. (Phelps, W.C. et al., In: Current Topics in Microbiology and Immunology: Transforming Proteins of DNA Tumor Viruses, Knippers, R. and Levine, A.J., Eds., Springer-Verlag, pp. 153-166 (1989)). The figure is a schematic representation of the E7 target sequence in the adenovirus E2 promoter linked to the CAT gene. Bal31 deletions with end points at -97, -79, -70 and -59 derived from the -285 to +40 AdE2CAT plasmid were generated. Five micrograms of each Ad E2CAT plasmid together with 5  $\mu$ g of either PBR322, PE1A (a plasmid encoding adenovirus E1a product) or p858 (a construct expressing the papillomavirus E7 product) were cotransfected into CV1 monkey cells and assayed for CAT activity. The locations of the E2F binding sites and its major and minor RNA initiation sites are shown.

- 2) Comparison of the amino acid sequences of HPV-16 E7 and adenovirus E1a proteins reveals regions of significant amino acid similarity which are well conserved within the E7 proteins of other papillomaviruses present in genital tissues.

Figure 2, taken from Phelps et al., (ibid), shows structural similarity between Adenovirus E1a and HPV-16 E7. A schematic diagram of the Ad5 E1a region is shown at the

top of the figure with the conserved amino acid domains 1-3 indicated. Homologous (boxed) and functional (hatched) amino acids of domains 1 and 2 from E1a which are found in HPV-16 E7 are located within the N-terminal 37 residues. The predicted amino acid sequences for this region of the E7 proteins of the other genital-associated HPVs are shown.

- 3) The conserved sequences between E1a and E7 mentioned above are restricted to domains 1 and 2 of E1a. Those sequences are important for the ability of E1a to transform and induce DNA synthesis. (Lillie, J.W. et al., Cell 46: 1043-1051 (1986)).
- 4) Both E1a and E7 associate with the anti-oncogene product of the retinoblastoma (Rb) gene (Dyson, N. et al., Science 243: 934-937 (1989)). The conserved sequences between the two proteins which are important for transformation are also important for the association with the Rb protein. This suggests that the two proteins interact with the same cellular metabolic events to cause transformation.
- 5) E7 can complement the ras oncogene in transforming baby rat kidney cells in a similar fashion to E1a complementation of the ras oncogene (Phelps et al., ibid).

Thus, two DNA tumor viruses, each belonging to a different class of DNA viruses, have been shown to have structural and sequence similarities and to

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modulate host gene expression. Both E7 and E1a appear to possess multiple functions and it is reasonable to assume that their ability to modulate host gene expression is an important event in their ability to induce cellular transformation. If the cellular gene products which are targets for such oncogenic regulators which require the transforming domains of these DNA tumor viruses were identified, it would not only provide great insight into metabolic events which are affected during transformations, but also provide a means by which transformation by such DNA tumor viruses can be inhibited. In addition, it would make it possible to design markers useful in diagnosing infection with the responsible DNA virus and in monitoring subsequent transformation. It would also make it possible to design compounds or drugs useful for inhibiting activated targets or activation events, which could serve as chemotherapeutic drugs.

As described in the following sections, it has been determined that at least one cellular enzyme which is involved in energy metabolism and purine pathways (i.e., creatine kinase) and which is a tumor marker is a reasonable target of DNA tumor viruses. The oncogene proteins of the DNA tumor viruses act on host/cellular genes and modify expression of some selected genes, resulting in induction of a transformed state.

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Identification of Cellular Gene Products Which are  
Targets for Oncogenic Regulators

It is reasonable to expect that a cellular gene product, creatine kinase, which is an enzyme involved in maintenance of ATP at sites of cellular work or energy production, is a target of oncogenic products of DNA tumor viruses, such as the HPV E7 product and the adenovirus E1a product, which act as regulators and modify or regulate expression of cellular genes. In addition to discrete DNA tumor viruses, there appear to be factors which are consistently present in or secreted from some transformed cells or tumor cells, which in and of themselves are capable of inducing DNA synthesis in host cells and probably are able to modulate cellular gene expression with much the same effect that a DNA tumor virus has (i.e., increase in expression of the cellular gene). Such factors are referred to herein as DNA tumor virus factors.

Creatine kinase has been shown to be a target for phosphorylation by protein kinase C and tumor promoters. It is highly responsive to a variety of hormonal signals and has been associated with signal transduction mechanisms. Creatine kinase is elevated in a variety of tumors and has been described as a tumor-associated marker. For example, creatine kinase is used as a diagnostic marker for small cell lung carcinoma (Gazdar, A.F. et al., Cancer Res., 41:2773-2777 (1981)). High levels of creatine kinase are found in breast and prostate carcinomas, especially in the presence of

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overt metastasis (Silverman, L.M. et al., Clin. Chem., 25:1432-1435 (1979); Homberger, et al., Clin. Chem. 26: 1821-1824 (1980); Thompson et al., Lancet 98: 673-675 (1980)). In breast cancer, the tumor burden correlates well with the degree of elevation of brain creatine kinase. In cancers of the bladder, prostate, testis, head and neck, raised serum creatine kinase B levels occurred more frequently in patients with metastatic disease than in those thought to have local disease alone, while in sarcoma, cancer of the ovary, uterus, cervix, stomach, bowel and anal canal, the presence of persistent disease correlated with high serum CKB levels (Rubery, E.D. et al., Eur. J. Cancer Clin. Oncol., 18:951-956 (1982)). In neuroblastomas, the extent of the disease was associated with an increased incidence of elevated serum creatine kinase B. The highest pre-treatment blood levels were found in stage IV disease, and a strong correlation between the pretreatment CKB level and the outcome of the disease in patients with neuroblastomas was observed (Ishiguro, Y. et al., Cancer 65: 2014-2019 (1990)).

It is clear that CKB is an enzyme that is very active in many tumors, particularly those of epithelial origin (e.g., small cell carcinoma, neuroblastomas retinoblastomas, cervical carcinomas, bladder cancer, and ovarian cancer). Some of these malignancies may be triggered by a DNA tumor virus or DNA tumor virus factor. Alternatively, transformation could result from the action of other

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factors (e.g., cellular factor, growth factor), which have an effect on cells equivalent to that of DNA tumor virus or virus factor. For example, activated oncogenes, mutated tumor suppressors or anti-oncogenes, or autocrine growth factors may also act to increase purine metabolic enzyme activity. The present method of inhibiting growth, transformation, and/or metastasis of mammalian cells, is applicable to cells transformed by diverse mechanisms in which the activity of at least one purine metabolic enzyme is elevated.

As discussed below, creatine kinase has several characteristics which support the idea that it is such a target of oncogenic products of DNA tumor viruses. As is also discussed below, other enzymes which have a similar role in cellular metabolism (i.e., purine metabolic enzymes) and which work in association with CKB are likely to be targets of oncogenic products as well. Thus, interference with the ability of an oncogenic product of a DNA tumor virus, DNA tumor virus factor, or other factor which has an equivalent effect on cells to regulate expression of enzymes which participate in purine metabolism and/or interference with another enzyme(s) which interacts with the purine metabolic pathway enzyme(s) will result in inhibition of or interference with the ability of such viruses or factors to transform cells. This should be particularly useful in inhibiting tumor formation, growth or metastasis in epithelial cells and, particularly, in epithelial cells in which HPV is known to be

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present (e.g., cervical epithelial cells). The method of the present invention makes it possible to inhibit the ability of DNA tumor viruses to act as mediators of cell transformation, and thus, to inhibit cell transformation or tumor formation or spread, by acting upon production of viral DNA, RNA or encoded oncogenic products, as well as by interfering with other viral or cellular products which normally interact or cooperate with viral or cellular products to bring about cell transformation.

The gene encoding human brain creatine kinase (CKB) has been isolated and sequenced and its promoter has been shown to have a strong sequence relationship with the promoter region of the adenovirus E2E gene (Figure 3; Daouk, G.H. et al., J. Biol. Chem. 263(5): 2442-2446 (1988)). The E2E gene of adenovirus encodes a 72 Kd single stranded DNA binding protein which is involved in virus replication (Friefeld, B.R. et al., Virology, 124:380-389 (1983)). The expression of the E2E gene is highly dependent on the adenovirus oncogenic products of the E1a region (Berk, A.J., Ann. Rev. Genet., 20:45-79 (1986)). The first 95 bases of the E2E and CKB promoters are shown in Figure 3. Solid lines connect identical bases. This is the first example of a strong sequence similarity between a cellular gene promoter region and a viral gene promoter. It has also been shown that brain creatine kinase expression is regulated by the oncogenic products of the E1a region of the adenovirus. Thus,

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it has been shown that a cellular gene which is associated with energy metabolism and regulation of intracellular nucleotide levels is turned on, or activated, by the transforming region of an oncogene encoded by a DNA tumor virus. Induction of a cellular gene by oncogenic products has thus, been shown for the first time to require both of the transforming domains of the oncogenic protein of the virus (Kaddurah-Daouk et al., Mol. Cell. Biol. 19(4): 1476-1483 (1990)).

The unexpected sequence relationship between the E2E promoter to the CKB promoter region promoters prompted an investigation of a possible functional relationship between the two. Infection in tissue culture of a variety of human cell lines (e.g., cervical cells, monocytic cells and small cell carcinoma) has demonstrated that CKB expression is activated by adenovirus infection in every cell line tested that previously had a detectable CKB level. The E1a proteins of the oncogenic region are important for the induction, as is shown by the fact that infection with a deletion virus mutant dl312, which does not include the E1a region, is defective for induction.

What was striking was that domains one and two of the E1a molecule, which are associated with transformation and induction of DNA synthesis in host cells, are the same domains required for induction of CKB activity, as illustrated in Figures 4, 5 and 6. Figure 4 shows that infecting HeLa cells with a mutant virus (dl 1500) that expresses

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only the 12S product (associated with trans-formation) results in induction of CKB expression to an extent comparable to that seen when HeLa cells are infected with the 13S product, which encodes the entire protein with all three domains. Lanes a and b, mock infected plates probed with CKB probes; lanes c and d, RNA harvested from a dl 1500-infected plates (12S product producer) and probed with CKB probes; lanes e and f, results of probing RNA harvested from cells infected with pm975 virus (expressing 13S product).

Figure 5 shows that point mutations in transforming domain 2 are impaired for E1a-induced expression of CKB. HeLa cells were infected with different mutant viruses. In Figure 5A, the lanes are as follows: lane a, mock infection; lanes b, g, wild-type pm975 virus (13S product); lane c mutant dl 312 (an E1a deletion mutant); lanes d, e, h, i, mutant viruses pm 975-936 and 953, each of which has a point mutation in domain 2 and disrupts the ability of E1a to transform; lanes f, j, mutant virus pm 975-1098, which has a point mutation in domain 3 and does not affect transformation functions. Panel B shows results of probing the same RNA with adenovirus E2 probes.

Figure 6 shows that amino terminal deletions of E1a obliterate the induction of expression of CKB. HeLa cells were infected with amino terminal deleted viral constructs. Panel A shows creatine kinase activity, represented by bars. Panel B shows amino acids deleted in each mutant (right) and names given

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by Whyte and co-workers (Whyte, P. et al., J. Virol. 62: 257-265 (1988)). The same observation is seen when RNA harvested from cells is probed with CKB specific primers (data not shown).

Thus, the induction of CKB, which is a major enzyme in energy metabolism, regulation of nucleotide levels and purine pathways, associates well with the ability of E1a to transform. This leads to the conclusion that CKB induction must be part of the mechanism by which cells are transformed by at least one DNA tumor virus (adenovirus type 5).

In addition, as described above, the papilloma-viruses (e.g., type 16), which are associated with malignancies of the cervix, encode a protein (the E7-encoded protein) which is very similar in sequence and function to that of the E1a-encoded protein in the transforming region. In particular, several key similarities have been identified: (1) the E7-encoded protein has amino acid sequence homology to the E1a-encoded protein in the important transformation domain; (2) the E7-encoded protein, like the E1a-encoded protein can act to turn on the adenovirus E2 promoter; (3) both proteins interact with similar host proteins, such as the retinoblastoma gene product; and (4) the E7-encoded protein can replace the E1a-encoded protein in transformation assays in which complementation with ras is tested.

It is also important to note that the sequence of the adenovirus E2 promoter, which controls the replication of adenovirus, is very similar to that

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of the cellular CKB promoter. Either E1a of adenovirus or E7 of papillomavirus virus can induce the E2 promoter and they both require the first 90 bases of the promoter for induction. E1A turns on CKB expression and because the first 90 bp of the CKB promoter sequence are very similar to those of the E2 promoter, it is reasonable to expect that E7 acts similarly (i.e., turns on CKB expression). In addition, the fact that the transforming domains 1 and 2 of E1a are required for CKB induction by the adenovirus and the fact that the same sequences of the two domains are conserved in the E7 protein of papillomavirus strongly suggest that CKB will be induced by E7 and would require the transformation domains of E7. Mutations which disrupt the ability of E1a to transform are dysfunctional for CKB expression. These crucial amino acids are conserved in the transforming part of E7. It is likely that these two DNA tumor viruses cause some similar modifications in the host cell metabolism and hence work at least partially via common mechanisms to cause transformation. It has been shown that CKB associates with the ability of E1a to transform cells. It is reasonable, based on experimental proof and the literature, to predict that creatine kinase or the metabolic pathway is similarly affected in epithelial cells (e.g., epithelial cells of the cervix) which are infected by papilloma-viruses associated with malignancies. After infection of an epithelial cell by such a virus and expression by the virus of the encoded transforming

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proteins, increased expression of CKB and the enzymes working in conjunction with it (e.g., adenylate kinase and cyclase) occurs. Detection of some or all of these enzymes can serve as the basis for determining whether the virus is present and, if necessary, the extent to which it occurs.

Inhibition of Growth Factors Induced in Transformed or Virally Infected Epithelial Cells

For non-transformed mammalian cells to grow in vitro, they require the presence of appropriate polypeptide growth factor(s) that exert their effects through specific plasma membrane receptors. Primary epithelial cells are hard to grow in culture. This has hindered the study of epithelial cell transformation in vitro, and explains why most in vitro transformation experiments have been done with fibroblasts, although malignant tumors of non-epithelial cell origin account for only 10-20% of human neoplasms. However, human and rodent primary epithelial cells can be transformed with adenovirus, a DNA tumor virus (Houweling, A.P. et al., Virology 105:537-550 (1980); Vander Elsen, P.J. et al., Virology 131:242-246 (1983); Whittaker, J.L. et al., Mol. Cell. Biol. 4:110-116 (1984)).

Adenoviruses normally infect quiescent epithelial cells and the expression of the adenovirus type 5, E1a 12S gene product enables primary rodent epithelial cells to proliferate in the presence or absence of serum (Quinlan, M.P. and T. Grodzicker, J. Virol. 61:673-682 (1987)). Thus,

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the 12S gene product provides a means of studying the changes involved in the immortalization and transformation of primary epithelial cells. The 12S gene is a member of the adenovirus Ela transcription unit. At early times, after infection, and in transformed cells, two Ela transcripts designated 13S', 12S' mRNAs are produced. These mRNAs are translated into proteins of 289 and 243 amino acids, respectively, that differ only by the presence of an additional internal 46 amino acids in the 289 amino acid protein.

The products of the Ela region can immortalize primary cells (Moran, E. et al., J. Virol. 57: 765-775 (1986)) and can cooperate with other viral genes and cellular oncogenes to transform primary rat cells (Ruley, H.E., Nature 304:602-606 (1983)). The Ela 12S protein seems to play a major role in the stimulation of cell proliferation responses. The 12S gene product is required for optimal virus production in growth arrested permissive cells, but not in actively growing cells (Spindler, K.R. et al., J. Virol. 53:742-750 (1985)). It induces cellular DNA synthesis and cell cycle progression in quiescent cells (Quinlan, M.P. and T. Grodzicker, J. Virol. 61:673-682 (1987); Spindler, K.R. et al., ibid); Stabel, S.P. et al., EMBO J. 4:2329-2336 (1985)). It can immortalize primary epithelial cells so that they retain many of their differential characteristics.

The 12S product has been shown to cause/trigger the production of a growth factor, when introduced

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into primary kidney quiescent cells. Such a DNA tumor virus factor is a substance produced by or associated with a DNA tumor virus in a transformed or tumor cell and is capable of inducing immortalization in the absence of the DNA tumor virus itself. For example, recently Quinlan et al. (Quinlan, M.P. et al., PNAS 84:3283-3287 (1987)), found that infection of primary baby rat kidney cells with an adenovirus variant that encodes only the 12S product results in production of a growth factor that stimulates primary epithelial cells to proliferate. Media from cells that were infected with this variant virus, was filtered (to remove intact virus) and fed to cells which never saw the virus. Results showed that this conditioned media was able to induce epithelial cell DNA synthesis and proliferation between 24 and 36 hours after addition. A growth factor seems to be secreted from the epithelial cells upon infection with the 12S virus. The factor can act in an autocrine fashion and has a large molecular weight. The growth factor appears to be a unique mitogen for epithelial cells. Although it has been shown that the 12S product of adenovirus can induce DNA synthesis, proliferation, immortalization or transformation of its host cell, it is unclear how induction occurs. Other DNA tumor viruses associated with malignancies show sequence similarity to the 12S protein of adenovirus and most probably secrete similar factors.

It appears that the growth factor released by 12S adenovirus infection might be mediating the

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observed effects of the 12S product (immortalization, induction of DNA synthesis). As described previously, the 12S product is capable of inducing the expression of CKB. The regions required to induce CKB expression or the production of the viral factor are similar. This suggests that the virus might be affecting the host cell genes via the secreted factor. Hence, the factor which is the hormone might be the real inducer of CKB and other enzymes. Adjacent epithelial cells contacted by these factors may be stimulated to elevate purine metabolic enzyme activity, while undergoing DNA synthesis and proliferation. These cells may become transformed ultimately, or may secrete factors, enzymes or metabolites that are used by the original transformed cell. This model is further favored by the fact that the kinetics of induction of CKB and the factor are similar. It is reasonable to expect that this factor (or derivatives thereof) is secreted by many tumors in which CKB (and adenosine metabolic enzymes) is highly activated. Further support for this idea is the fact that many DNA tumor viruses, such as polyoma and papilloma virus encode transforming products with amino acid sequences similar to the amino acid sequence of the adenovirus 12S products. Thus, cells infected with such viruses are very likely going to express this DNA tumor virus factor or transforming factor and cause induction of purine metabolic pathway enzymes in much the same manner as the 12S factor causes induction.

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The CKB gene is known to be very responsive to multihormonal signals, implying it is indeed active in signalling or is along a metabolic path that mediates these effects. Thus, this adenovirus factor might be a new hormone which also regulates CKB. Most of these hormones, in spite of being different and having different receptors, might pass by or affect a common event which CKB is part of.

A seemingly unrelated virus, the cytomegalovirus, which induces CKB expression (Colberg-Poley, A.M. et al., Virology, 166(1):217-228 (1988)), also appears to induce production of a factor capable of stimulating DNA synthesis and modifying cell growth (Gonczol, E. and S. A. Plotkin, J. Gen. Virol., 65:1833-1834 (1984)). This factor appears to function, at least partially, by modifying microtubule structure. Creatine kinase is thought to associate with the microtubule structure (Eckert, B.S. et al., J. Cell. Biol. 86: 1-5 (1980)). This factor might be the inducer of CKB expression.

Interruption of the autocrine loop which links this growth factor and its induction of enzymes, using selected drugs (e.g., competitive factor antagonists) can be carried out, using competitive antagonists which interfere with effects of the factor. Drugs may also interfere with the ability of the factor to act on cells, blocking autocrine stimulation or stimulation of adjacent cells. In addition, antibodies against the factor or receptor it interacts with can be used to block the factor's

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effect. Such compounds can be used for the treatment of virally infected cells and tumors of epithelial origin.

#### Uses of the Present Method

Because of the increased levels of CKB and other cooperative enzymes, simple enzyme assays can be used to detect a DNA tumor virus in cells in which one is thought to be present. Such assays can also be used for diagnostic purposes or for monitoring treatment provided to an individual in which the presence of transformed cells has been detected. Enzyme assays may also be used together with other methods of diagnosis. For example, the sensitivity of cytologic examination (Papanicolaou smear test) carried out in conjunction with detection of human papillomavirus (HPV) may be superior to the use of either cytologic studies or HPV detection alone in evaluating patients with cervical lesions (Ritter, D.B. et al., Am. J. Obst. Gynecol., 159(6): 1517-1525 (1988)). However, presently-available methods for detection of HPV, such as Southern blots which use labelled probes from the virus, are often technically difficult, time consuming, or costly. In contrast, enzyme assays are practical, rapid (e.g., in this case, approximately 25 minutes), inexpensive and easy to carry out in clinical settings. The detection of infection with HPV, through the method of the present invention, can be substituted for Southern blotting or other technically difficult, time

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consuming or costly methods, as a routine assay to complement the Papanicolaou smear test.

For example, a diagnostic assay can be carried out as follows: proteins can be extracted from cervical scrapings, such as those obtained for cytologic evaluation and extracted (e.g., by freeze-thawing). The supernatant can be assayed for enzymes of interest (e.g., creatine kinase, adenylate kinase). Two or more enzymes can be assessed simultaneously. Such enzyme assays can measure, for example, CKB activity, as well as activity of one or two additional enzymes along the purine metabolic pathway.

A drug or compound suitable for use in the method of the present invention is one which: (1) is able to affect cells in which a DNA tumor virus, DNA tumor virus factor, or other factor which has an equivalent effect on cells has acted to cause, directly or indirectly, an elevation of the activity of a purine metabolic enzyme(s) and (2) is able to prevent the transforming domain of the oncogenic virus, tumor virus factor or other factor which has an equivalent effect on cells from inducing the purine metabolic activity or is able to inhibit the activity of the purine metabolic enzyme, directly or indirectly. The drugs can act outside of the cell, (e.g., by blocking the effect of a secreted factor or enzyme, by influencing uptake of creatine), or can enter the cell to act within the cell in one or more of the above-described ways. A drug having these characteristics can be a protein, peptide, a

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nucleic acid sequence, or an agent which acts, directly or indirectly, to reduce the velocity of a purine metabolic enzyme. For example, inhibitors, substrate analogs, or slow substrates of a purine metabolic enzyme will, when present with the natural substrate(s), reduce the velocity of the enzymatic reaction catalyzed by a purine metabolic enzyme on the natural substrate(s). Note that preventing induction of a purine metabolic enzyme(s) in a cell in which its activity is elevated will also reduce the velocity of reaction. Drugs useful in the present method can be selected or designed to act in one or more of the above-described ways.

The drug(s) is administered to an individual in whom a DNA tumor virus or virus factor has acted, resulting in a cellular abnormality or a tumor. The drug is administered in sufficient quantity to reduce or eliminate the effect of the DNA tumor virus, virus factor, or other factor on cells.

For example, a drug which is an antisense construct (e.g., oligonucleotide sequence) which binds selectively to a region of the RNA necessary for translation can be used. In the case of CKB, the oligonucleotide sequence is one which binds selectively to a region of CKB RNA, as represented in Figure 7, which is needed for translation. The effects of the transforming viral DNA are, as a result, reduced or prevented. That is, an oligonucleotide sequence capable of entering the cell and binding (hybridizing) to the specific activated message to block it can be introduced into

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cells. Hybridization to the nucleotide sequence renders it unavailable for further activity. In another approach, a drug which interferes with a transcription factor (e.g., by acting upon a promoter or blocking the ability of a transcription factor(s) to activate a promoter(s)) is administered in sufficient quantities to have the desired effect. Alternatively, a drug which inhibits interaction of the viral oncogenic product(s) (e.g., the E7 product, E1a product) with a cellular gene encoding a purine metabolic enzyme can be administered. Inactivation can occur, for example, by binding of the drug to the viral oncogenic product or by destruction of the viral oncogenic product by the drug.

Drugs which act to inhibit the activity of a purine metabolic enzyme(s) which is elevated in transformed cells may act directly or indirectly. Inhibition will result in a decrease in the activity of the purine metabolic enzyme(s), and of the velocity of the reaction in particular. For example, the drugs may act by interfering with the ability of purine metabolic enzymes to associate or interact with one another, or by altering the activity of an enzyme in the pathway resulting in decreased velocity of the purine metabolic enzyme whose activity is elevated.

The particular target is determined by the activity elevated in the cell. For example, the cellular gene encoding the purine metabolic enzyme adenosine deaminase has been shown to have a

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striking resemblance (sequence similarity) at the promoter level to brain creatine kinase. Preliminary data shows that the adenosine deaminase promoter, like the promoter of brain creatine kinase, is induced by the E1a products. This is of particular interest because adenosine deaminase is also a tumor antigen/thymus leukemia antigen, is involved in adenosine metabolism, and is vital for DNA synthesis. By use of the present invention, transformation, growth and/or metastasis of cells in which the activity of adenosine deaminase is elevated could be inhibited.

Adenylate kinase is another purine metabolic enzyme which can be inhibited by the present method and whose inhibition may result in inhibition of cellular transformation. Adenylate kinases (NTP: AMP phosphotransferases, N, adenine or guanine) are relatively small (21-27 kD), monomeric enzymes which catalyze the interconversion of nucleotides according to the equation:  $Mg^{++} \text{ NTP} + \text{AMP} \rightleftharpoons Mg^{++} \text{ NDP} + \text{ADP}$ . The enzyme is ubiquitous, is abundant in tissues where the turnover of energy from adenine nucleotides is high (Noda, L., In: Enzymes, (Boyer, P., ed.) Vol. 8, pp. 279-305, Academic Press, Orlando, Fl), and has an important role in maintaining energy charge of the adenylate pool (Lipman, F., Curr. Top Cell. Regul., 18:301-311 (1981); Atkinson, D.E., Biochemistry, 7:4030-4034 (1968)). The prokaryotic gene from E. coli has been cloned (Brune, M. et al., Nucleic Acids Res., 13:7139-7151 (1985). This and previous studies

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(Glaser, M. et al., J. Bacteriol., 123:128-136 (1975)) have shown that adenylate kinase is a key enzyme in controlling the rate of cell growth. By the method of the present invention, the activity of adenylate kinase in tumor cells in which adenylate kinase is elevated may be inhibited. Furthermore, by virtue of its association with creatine kinase and/or because it can provide dinucleotide substrates for creatine kinase, inhibiting the activity of adenylate kinase is expected to decrease the velocity of creatine kinase.

The isozymes of creatine kinase catalyze the resynthesis of ATP for use by cellular ATPases, processes of contraction, macromolecular synthesis, and maintenance of ion gradients, for example (Bessman, S.P. and C.L. Carpenter, Ann. Rev. Biochem., 54:831-62, (1985)). Creatine, creatine phosphate and creatine kinase have been proposed to facilitate energy distribution in several tissues, such as muscle, heart and brain by means of the creatine phosphate shuttle.

There are many possible sites for the action of drugs that inhibit brain creatine kinase and/or other enzymes which participate in purine metabolism. Thus the effects of such drugs can be direct or indirect, operating by mechanisms including, but not limited to influencing the uptake or biosynthesis of creatine, the function of the creatine phosphate shuttle, inhibiting the enzyme activity, or the activity of associated enzymes, or

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altering the levels of substrates or products of a reaction, to alter the velocity of the reaction.

In the method of the present invention, at least one drug which is capable of inhibiting the induction or activity of a purine metabolic enzyme (e.g., adenylate kinase or creatine kinase) or another enzyme which participates in the purine metabolic pathway, or a cellular component which cooperates with a purine metabolic pathway enzyme is administered to an individual in a manner appropriate for introducing the drug into affected cells. A drug of the present invention may also be administered in combination with other drugs known to be effective in treatment or with one or more additional drugs of the present invention. One or more drugs affecting a single target or affecting one or more targets in the pathway may be administered to increase the effectiveness of inhibition. The form in which the drug(s) will be administered is determined by the type of drug used, as will the route of administration and the quantity given.

For example, in the case of an individual in whom cervical dysplasia or cervical carcinoma is present, a drug which is able to inhibit the activity of the HPV oncogenic products and, thus, inhibit modulation of the cellular gene (e.g., the CK gene, adenylate kinase gene) is administered in a therapeutically effective quantity. Such a drug can be, for example, a peptide which binds domains 1 and/or 2 of E1a or E7, to prevent them from inducing

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their transactivation activity. Alternatively or in addition, inhibitors of the activated cellular enzymes are administered. In this instance, as well as in other instances of epithelial tumors, the drug(s) can be administered topically or can be injected, infused or otherwise introduced, alone or in combination with other drugs of the present invention or other known therapies.

A further application of the work described herein is identification of existing compounds, analogues of such compounds or new drugs capable of inhibiting or counteracting the effects of DNA tumor viruses, tumor virus factors, or other factors with an equivalent effect on cells. For example, tumor cells which display an elevation of purine metabolic enzyme activity can be exposed in culture to a drug candidate, and then assayed for growth by the incorporation of tritiated thymidine into DNA. Drugs that cause a decrease in growth relative to the untreated control in such an assay or drug candidates which decrease the activity of purine metabolic enzymes in assays in vitro may be used to inhibit growth, transformation, or metastasis by the method of the present invention. A variety of presently-available drugs (described below) can also be assessed for their ability to inhibit creatine kinase or adenylate kinase, which seems to work in conjunction with creatine kinase and can serve as models for design of additional drugs for creatine kinase inhibition.

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The sensitivity of tumor cell lines to inhibition of growth by cyclocreatine appears to correlate with creatine kinase activity. In particular, as shown herein, the more CK activity present in the cell, the more sensitive the cell is to the antiproliferative effects of cyclocreatine. This observation suggests that these and other tumor types having high creatine kinase activity or having creatine kinase activity which is elevated relative to that of the corresponding normal cell type, will be susceptible to inhibition by cyclocreatine (sensitive to cyclocreatine). Thus, the invention further relates to a method of identifying tumors which will be amenable to treatment with cyclocreatine (i.e., which are sensitive to cyclocreatine). In the method, the creatine kinase activity of a tumor specimen and/or a serum sample as appropriate is determined. Tumor samples having high creatine kinase activity, or having creatine kinase activity which is elevated relative to that of a corresponding normal cell type (i.e., a normal sample), are expected to be sensitive to cyclocreatine. Similarly, elevated serum creatine kinase levels (compared to a serum levels in a normal individual or normal baseline value) can be indicative of sensitivity to cyclocreatine of a tumor (e.g., primary, metastatic).

Drugs useful in the present method can be existing substances, analogues of existing substances or substances designed specifically to interfere with the action or effects of DNA tumor

viruses, DNA tumor virus factors, or other factors which have an equivalent effect on cells. The following is a description of existing substances known to inhibit creatine kinase, adenylate kinase or adenosine kinase. It will be possible to modify the substances described below to produce analogues which have enhanced characteristics, such as greater specificity for the enzyme or the oncogenic product, enhanced stability, enhanced uptake into cells, tighter binding to the enzyme or the oncogenic product or better inhibitory activity. In addition, based on knowledge of the structural and functional characteristics of the oncogenes and of the cellular genes upon which the oncogenic products act, it is possible to design other drugs useful in the present method.

Compositions Useful in the Present Method of  
Inhibiting Purine Metabolic Enzymes

Compounds which inhibit CKB and/or other enzymes which participate in purine metabolism, directly or indirectly (e.g., by interacting with enzymes in the pathway) to reduce the velocity of the enzyme will have great value in preventing and/or treating tumors characterized by elevated levels of these enzymes (e.g., cervical tumors). Such tumor cells may require elevated levels of CKB and/or other enzymes which participate in purine metabolism to meet their high energy demands for growth and metastasis. An advantage of drugs that target pathways that seem to be preferentially

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required by tumor cells, is that they may be specifically toxic to tumor tissues, and thus may lack the general toxicity associated with less specific drugs.

These drugs can be inhibitors (e.g., irreversible inhibitors), substrate analogs, bi-substrate analogs, transition state analogs and/or slow substrates of a purine metabolic enzyme, which when present, reduce the velocity of the enzymatic reaction catalyzed by a purine metabolic enzyme on the natural substrate(s). For example, a transition state analog covalent inhibitor can be applied locally (e.g., in the cervix). In addition, inhibitors of the enzymes that work in conjunction with CKB can now be designed and used, individually, in combination or in addition to other drugs, to make control of the effect on CKB tighter. For example, inhibitors of adenylate kinase, an enzyme that physically and functionally associates with creatine kinase, can be designed. Such drugs, alone or in combination, are useful as antitumor agents.

The following are drugs which can be used, alone, in combination, or in combination with existing therapies in the method of the present invention in order to inhibit the purine metabolic enzyme indicated. These drugs, modifications of these drugs, and new drugs can also be used. Suggestions of specific modifications and strategies for designing analogs are presented. It is to be understood that these are just examples of drugs

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which can be used in the present method and that this is not intended to be limiting in any way.

### Inhibitors of Creatine Kinase

#### Drugs (antitumor, antiproliferative)

The pathways of biosynthesis and metabolism of creatine and creatine phosphate can be targeted in selecting and designing drugs which may reduce the velocity of the reaction catalyzed by creatine kinase. Drugs targeted to specific steps may rely on structural analogies with either creatine or its precursors. Novel creatine analogs differing from creatine by substitution, chain extension, and/or cyclization may be designed. The substrates of multisubstrate enzymes may be covalently linked, or analogs which mimic portions of the different substrates may be designed. Specifically, the inhibition of the creatine kinase reaction may be facilitated by a covalent link between adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), or an analog of either, and creatine, or an analog, through the reactive termini of the molecules or via a spacer, such as  $(CH_2)_n$ :

- |    |                |                |
|----|----------------|----------------|
| a) | Creatine-ADP   | Creatine-ATP   |
| b) | Creatine-R-ADP | Creatine-R-ATP |

where R is a spacer, such as  $(CH_2)_n$ .

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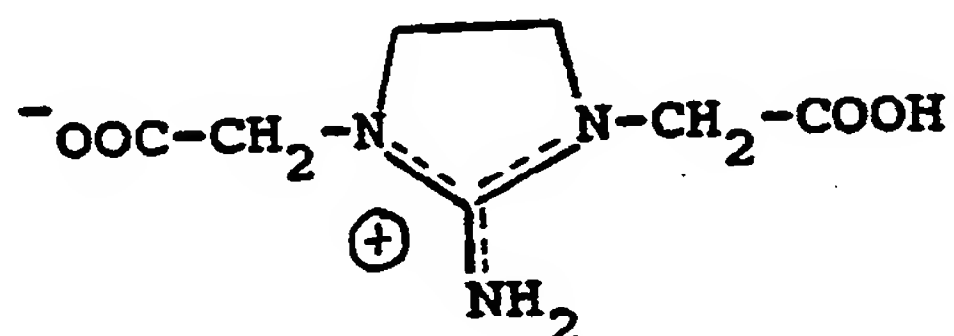
Replacement of the phosphate backbone or portions of the backbone with methylene groups may facilitate uptake by cells or increase stability. Variations which reduce the net charge of such an analog may also facilitate uptake by cells.

Additional Examples:

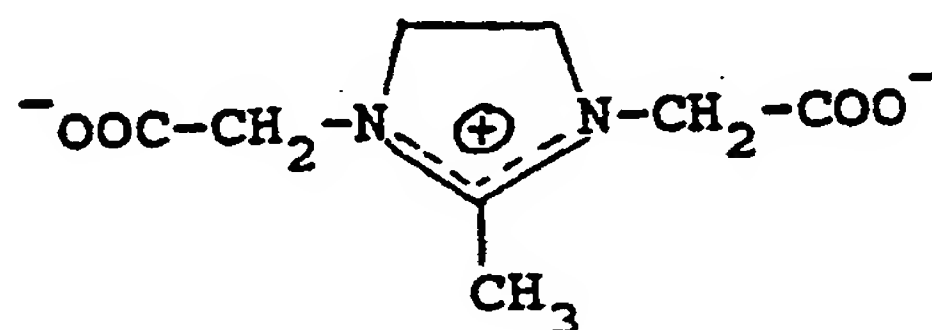
- 1) Inhibitors of creatine kinase and analogs of creatine described by Kenyon and co-workers (Rowley, G.L. et al., J. Am. Chem. Soc., 93:5542-5551, (1971); McLaughlin, A.C. and M. Cohn, J. Biol. Chem., 247:4382-4388, (1972); Nguyen, A.C.K., "Synthesis and enzyme studies using creatine analogs", Thesis, Dept. of Pharmaceutical Chemistry, Univ. Calif., San Francisco, (1983)) and others (Lowe, G. and Sproat, B.S., J. Biol. Chem., 255:3944-3951, (1980)); variants with appropriate modifications to enhance activity or uptake by cells.

## Examples

- a) 1,3 Dicarboxy-methyl-2-imino-imidazolidine



- b) 2-Methyl-N,N'-dicarboxymethyl-imidazole



- c) Epoxycreatine

- 2) Compounds described by Walker and co-workers to inhibit creatine kinase (Roberts, J.J. and Walker, J.B., J. Biol. Chem., 260:13502-13508 (1985)).
- 3) Small organic molecules which inhibit the purine metabolic enzyme, such as:
- butanediones  
(e.g., 2,3-butanedione)
  - cyclopropane derivatives
  - salicylate derivatives  
(e.g., iodoacetamidosalicylate)

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- iodoacetamide derivatives  
(e.g., N-(iodoacetamidoethyl)amino  
naphthalene-1-sulphonate,  
N-(4-iodoacetamidophenyl)amino  
naphthalene-1-sulphonate)
- benzene derivatives  
(e.g., 1-fluoro-2,4-dinitrobenzene,  
5,5'-dithiobis(2-nitrobenzoic acid))
- N-cyclohexyl-N-beta-methyl morpholinium salts

Derivatives or analogues of these molecules which will make them specific to CKB by linking them covalently to the substrates of CKB (i.e., creatine or ADP/ATP).

4) Derivatives of ADP, ATP, such as:

- a) 2',3'-dialdehyde derivatives of ADP, ATP;
- b) ATP- $\gamma$  (N-(2-chloroethyl)-N-methyl) amide  
(a mustard derivative);
- c) imidazolides of AMP, ADP, ATP.

5) A natural inhibitor in the serum of some patients, such as those with muscular dystrophy (which is a small dialyzable molecule of still unidentified origin).

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- 6) Compounds which uncouple of creatine kinase from the complex: adenylate kinase/translocase/creatine kinase.
- 7) Some of the blockers used in resolving a myocardial infarction are thought to inhibit creatine kinase.

#### Inhibitors of Adenylate Kinase

- 1) Multi-substrate inhibitors (Valentine, N., et al., Am. J. Hematol., 32(2):143-145 (1989); Gustav, E., et al., J. Biol. Chem. 248:1121-1123 (1973)).
  - a)  $P^1, P^5$ -Di(adenosine-5')pentaphosphate
  - b) The above compound in which methylene groups replace some or all of the phosphate groups, to facilitate uptake by cells.
  - c)  $AP_4A$ ,  $P_1, P_4$ -(diadenosine-5')tetraphosphate and its methylene substitutions.
- 2) Elemental sulfur and its derivatives (Conner, J. and P.J. Russel, Research Communications, 113(1):348-352 (1983)).
- 3) Adriablastin

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(Toleikis, A.I. et al., Biokhimiya,  
53(4):649-654 (1988)).

- 4) 5,5'-dithiobis-2(nitrobenzoate)  
(Declerck, P.J. and M. Muller, Comp. Biochem. & Physiol., 88(2):575-586 (1987)).
- 5) Adenosine, di-, tri-, tetraphosphopyridoxals  
(Yasami, T., et al., FEBS Lett., 229(2):261-264 (1988)).
- 6) Potassium ferrate  
(Crivellone, M.D., et al., J. Biol. Chem.,  
260(5):2657-2661 (1985)).
- 7) 8-Azido-2'-O-dansyl-ATP  
(Chuan, H., et al., J. Bio. Chem.,  
264(14):7981-7988 (1989)).

#### Inhibitors of Adenosine Kinase

- 1) 5-iodotubercidin (from sponge of genus  
Echinodictyum)  
(Weinberg, J.M., et al., Am. J. Physiol.,  
254(3p+2) pF311-322, (1988)).
- 2) Two very potent inhibitors from marine sources  
4-Amino-5-bromo-pyrrolo[2,3-d] pyrimidine  
  
5'-Deoxy-5-iodotubercidin  
(from red alga Hypnea Valentiae)

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(Davies, L.P., et al., Biochem. Pharm.,  
33(3):347-355 (1984)).

- 3) Clitocine and its derivatives  
(Moss, R.J., et al., J. Med. Chem.,  
31(4):786-790 (1988)).

A link between transformation by DNA tumor viruses and the increased activity and expression of the brain isozyme of creatine kinase (CKB) has been demonstrated, suggesting that high levels of CKB are needed for the biochemical events triggered during oncogenesis. Alterations in energy metabolism or signal transduction mediated by CKB may be playing an active role in these malignancies. A panel of transformed and non-transformed cell lines were established in culture and were tested for creatine kinase activity. Cell lines which displayed high creatine kinase activity were then analyzed to determine the level of the brain isozyme of creatine kinase (CKBB, see Example 1). Many transformed cells expressed high levels of brain specific creatine kinase.

A series of 15 creatine analogs or guanidino compounds (including inhibitors of creatine kinase and/or slow substrates, which, in the presence of the natural substrate(s), act to reduce the velocity of the reaction catalyzed by creatine kinase on its natural substrate) were synthesized or purchased and studied for their effects on the growth of several

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tumor cell lines, especially on those which have high levels of CKB.

As shown in Example 2, five of the creatine analogs inhibited the growth of tumor cell lines in culture. The drugs, homocyclocreatine, cyclocreatine, 1-carboxymethyl-2-imino-hexahydropyrimidine, guanidinoacetate and carbocreatine, which are slow substrates for creatine, showed different patterns of activity against a panel of cell lines. Cyclocreatine, the only analog tested which is phosphorylated efficiently by creatine kinase, was the only creatine analog that consistently showed anti-proliferative activity specifically against cell lines expressing high levels of CKB. Several additional preparations of cyclocreatine were synthesized, and then further purified by a series of recrystallizations. These preparations retained a similar level of anti-proliferative activity.

The patterns of activity for homocyclocreatine and 1-carboxymethyl-2-imino-hexahydropyrimidine were similar in that the cell lines affected were the same and the degrees of inhibition by either drug were similar. The latter two drugs preferentially inhibited the growth of the prostate and cervical cell lines which are metastatic in origin as compared with nonmetastatic prostate and cervical cell lines. Furthermore, two non-transformed cell lines, MRC-5 and Vero, which are tissue culture versions of "normal" cell lines, were largely unaffected by these two drugs, suggesting that they may spare normal cells and may have a low toxicity

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in vivo. However, the active component of these preparations is undefined, as both preparations have lost some anti-proliferative activity following four successive recrystallizations.

Guanidinoacetate strongly inhibited the growth of three colon tumor cell lines tested. (The data for guanidinoacetic acid is from two separate experiments and as such is not as extensive as that for the other three drugs.) The specificity of the homocyclocreatine preparation, the 1-carboxymethyl-2-iminohexahydropyrimidine preparation, and cyclocreatine for certain cell lines suggests that these drugs lack the indiscriminate toxicity observed with other anti-tumor therapies. In contrast, carbocreatine exhibited apparent non-specificity under the conditions of these studies. In addition, under the conditions used in the assay, and on the specific cell lines tested, ten other compounds (see Table 4) showed little or no activity (Example 3).

The present invention will be further illustrated by the following examples, which are not intended to be limiting in any way. The Materials and Methods following immediately were used in Examples 1-6.

### Materials and Methods

#### Compounds

Compounds prepared for use in these studies included homocyclocreatine (1-carboxyethyl-2-iminoimidazolidine), cyclocreatine

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(1-carboxymethyl-2-iminoimidazolidine), 1-carboxymethyl-2-iminohexahydropyrimidine, guanidinoacetate (glycocyamine) and carbocreatine. The synthesis of the homocyclocreatine preparation was carried out as described by Roberts and Walker (Roberts, J.J. and J.B. Walker, Arch. Biochem. Biophys., 220:563-571 (1983)). The syntheses of cyclocreatine and the 1-carboxymethyl-2-iminohexahydropyrimidine preparation were carried out as described by Griffiths and Walker (Griffiths, G.G. and J.B. Walker, J. Biol. Chem., 251:2049-2054 (1976)). Guanidinoacetate was obtained from Sigma Chemical Corp. (St. Louis, MO). The synthesis of carbocreatine was carried out as described (Nguyen, Ann Cae Khue, Ph.D. Thesis in Pharmaceutical Chemistry, University of California, San Francisco, CA, 1983).

#### Tumor Cell Lines:

A variety of tumor cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The neuroendocrine-derived tumor cell lines, such as the small cell lung carcinoma cell line, were grown as cell suspensions in flasks and were replated and fed twice a week. The other cell lines were grown attached on plastic plates and were replated at 90% confluency. All cell lines were grown in media supplemented with fetal bovine serum.

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The properties of the cell lines used in these studies have been described (American Type Culture Collection (ATCC) Catalogue of Cell Lines and Hybridomas, 6th edition, Hay et al., Eds., 1988). Some of the the properties of the cell lines, including the origin, tumorigenicity, and markers, are summarized below.

Prostate Cell Lines

DU145: Prostate carcinoma, metastasis to brain,  
Human

Eagle's MEM, 90%; Fetal Bovine Serum (FBS), 10%

Isolated from a lesion in the brain of a patient with widespread metastatic carcinoma of the prostate and a three year history of lymphocytic leukemia. The cells have an epithelial-like morphology, are weakly positive for acid phosphatase, are not hormone sensitive, and form colonies in soft agar. Tumorigenic in nude mice. (Mickey, D.C. et al., Cancer Res. 37: 4049-4058 (1977); Stone, K.R. et al., Int. J. Cancer, 21: 274-281 (1978)).

LNCaP.FGC: Prostate adenocarcinoma, metastasis to  
lymph node, Human

RPMI 1640, 90%; FBS, 10%

Isolated from a needle aspiration biopsy of the left supraclavicular lymph node of a 50 year-old male with metastatic carcinoma of the prostate. The

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cell line produces prostatic acid phosphatase and prostate specific antigen, and has androgen receptors. Tumorigenic in nude mice. (Models of Prostate Cancer, G.P. Murphy (ed.), pp. 115-132, Alan R. Liss, Inc., New York, 1980; Cancer Res. 43: 1809-1818, (1983); Cancer Genet. Cytogenet. 11: 399-404 (1984)).

PC-3: Prostate adenocarcinoma, Human  
Eagle's MEM, 90%; FBS, 10%

Isolated from a grade IV prostatic adenocarcinoma from a 62 year-old male. The cell line has low acid phosphatase activity, grows in soft agar, and can grow in suspension. Tumorigenic in nude mice. (Invest. Urology 17:16-23, (1979); Cancer Res. 40: 524-534, (1980)).

#### Colon Cell Lines

SW48: Colon adenocarcinoma, Human  
Leibovitz's L-15 Medium, 90%; FBS, 10%

Isolated from a grade IV colorectal adenocarcinoma, which encircled the bowel of an 82 year-old female. The cells have an epithelial-like morphology, and low levels of CEA. Tumorigenic in nude mice. (Leibovitz, A. et al., Cancer Res. 36: 4562-4569, (1976); Fogh, J. et al., J. Natl. Cancer Inst. 59: 221-226, (1977)).

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SW403: Colon adenocarcinoma, Human  
Leibovitz's L-15 Medium, 90%; FBS, 10%

Isolated from a grade III colorectal adenocarcinoma, which almost encircled the bowel of a 51 year-old female. The cells have an epithelial-like morphology, and produce carcinoembryonic antigen (CEA). Tumorigenic in nude mice. (Leibovitz, A. et al., Cancer Res. 36: 4562-4569, (1976); Fogh, J. et al., J. Nat. Cancer Inst. 59: 221-226 (1977)).

SW1116: Colon adenocarcinoma, Human  
Leibovitz's L-15 Medium, 90%; FBS, 10%

Isolated from a grade II adenocarcinoma of the colon, which extended into the muscularis of a 75 year-old male. Melanosis was observed. The cells have an epithelial-like morphology, show brush borders, and produce high levels of CEA. (Leibovitz, A. et al., Cancer Res. 36: 4562-4569, (1976)).

#### Cervical Cell lines

C-33A: Cervical carcinoma, Human  
Eagle's MEM (with non-essential amino acids and sodium pyruvate), 90%; FBS, 10%

Isolated from a cervical cancer biopsy from a 66 year-old female. The cells have an epithelial-like morphology. Tumorigenic in nude mice.

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(Auersperg, N., J. Natl. Cancer Inst. 32: 135-148, (1964)).

CaSki: Cervical epidermoid carcinoma, Human  
RPMI 1640, 90%; FBS, 10%

Isolated from an epidermoid carcinoma of the cervix metastatic to the mesentery of the small bowel of a 40 year-old patient. The cells express tumor-associated antigen and produce the beta subunit of human chorionic gonadotropin. (Pattillo, R.A. et al., Science 196: 1456-1458, (1977)).

SiHa: Cervical squamous carcinoma, Human  
Eagle's MEM (with non-essential amino acids and sodium pyruvate), 90%; FBS, 10%

Isolated from a primary tissue surgical sample from a 55 year-old patient. Epithelial-like morphology. Forms tumors in nude mice. (Friedl, F. et al., Proc. Soc. Exp. Biol. Med. 135: 543-545, (1979)).

HeLa: Cervical epitheloid carcinoma, Human  
Eagle's MEM with non-essential amino acids and Earle's BSS, 90%; FBS, 10%

Isolated from a cervical carcinoma of a 31 year-old (Cancer Res., 12: 264, (1952)).  
Epithelial-like morphology.

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ME-180: Cervical epidermoid carcinoma, Human  
McCoy's 5a medium, 90%; FBS, 10%

Isolated from an omental metastasis of a rapidly spreading cervical carcinoma from a 66 year-old (Sykes, et al., J. Nat. Cancer Inst. 45: 107-122, (1970)). The tumor was a highly invasive squamous cell carcinoma and shows epithelial-like morphology in culture. Tumorigenic in nude mice.

Lung Cell Lines

NCI-H69: Small cell carcinoma of the lung, Human  
RPMI 1640, 90%; FBS, 10%

Isolated from the pleural fluid of a 55 year-old male with small cell carcinoma of the lung. Forms colonies in soft agar and grows in suspension. Has small cell carcinoma morphology and APUD characteristics. Tumorigenic in nude mice. (Gazdar, A. et al., Cancer Res. 40: 3502-3507, (1980)).

MRC-5: Diploid non-transformed lung cell line,  
Human  
Eagle's Basal Medium with Hanks' BSS, 90%; FBS, 10%

Derived from normal lung tissue of a 14 week-old fetus. Fibroblast-like morphology. (Nature 277: 168-170, (1970)).

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Bone Cell Lines

U-2 OS: Primary osteogenic sarcoma, Human  
McCoy's 5a medium, 85%; FBS, 15%

Derived from a sarcoma from the tibia of a 15 year-old. Epithelial-like morphology (Int. J. Cancer, 2: 434-447, (1967)).

Saos-2: Primary osteogenic sarcoma, Human  
McCoy's 5a medium, 85%; FBS 15%

Isolated from an 11 year-old. This line has an epithelial-like morphology consistent with osteogenic sarcoma. The cells do not form tumors in nude mice. (In: Human Tumor Cells In Vitro, pp. 115-159, J. Fogh, (ed.), Plenum Press, New York, (1975))

Kidney Cell Lines

293: Transformed primary embryonal kidney, Human  
Eagle's MEM, 90%; horse serum, 10%

The 293 cell line was derived from primary human embryonal kidney cells transformed with sheared DNA from human adenovirus type 5. (J. Gen. Virol., 36: 59-72, (1977)).

Vero: Normal kidney, African Green Monkey  
Medium 199, 95%; FBS, 5%

Initiated from the kidney of a normal adult African Green Monkey (Yasumura, Y. and Kawakita, Y.,

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Nippon Rinsho, 21: 1209, (1963)). Fibroblast-like morphology.

Embryonal Cell Lines

BALB/3T3: Embryonal tissue, Mouse

Dulbecco's modified Eagle's medium, 90%; calf serum, 10%

Isolated from disaggregated mouse embryos. The line is non-tumorigenic, and shows contact inhibition. (Aaronson, S.A. and Todaro, G.T., J. Cell Physiol. 72: 141-148, (1968)).

F9: Embryonal carcinoma, Mouse

Dulbecco's modified Eagle's medium with 4.5 g/L glucose, 85%; FBS, 15%

Initiated from a testicular teratocarcinoma of a mouse (Bernstine et al., Proc. Natl. Acad. Sci. USA, 70: 3899-3903, (1973)). The cell line can be induced to differentiate into parietal endoderm under certain culture conditions.

Proliferation Assays:

The rate of incorporation of thymidine into DNA was adopted as a measure of cell proliferation. To test the effect of several creatine analogs (inhibitors of creatine kinase and/or slow substrates, referred to here as drugs), on the growth of cell lines, the cells were harvested and replated in either 24 or 96 well plates at an

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appropriate density. The next day, drugs were added at the indicated concentration. Tritiated thymidine (<sup>3</sup>H-thymidine; Amersham) was added to a final concentration of 2  $\mu$ Ci/ml one hour to twenty-four hours before the cells were harvested, depending on the length of the experiment. Treated cells were harvested by one of two methods. In the first method, cells were lysed by the addition of 10% trichloroacetic acid or 10% sodium dodecylsulfate. Precipitated DNA was removed by centrifugation and was blotted onto 3MM filter paper. Filters were washed sequentially with methanol and acetone, dried, then placed in vials containing Ready Safe liquid scintillant (Beckman) and counted in a Beckman LS 31-33 T liquid scintillation counter.

In the second method, media was removed by aspiration or inversion and the cells were incubated for five minutes at 37°C in 100  $\mu$ l of trypsin (2.5%, Sigma Chemical Corp., St. Louis, MO) to detach the cells. Cells were harvested with a Skatron cell harvester, collected on 102 x 258 mm glass fiber filter paper (Pharmacia #1205-401), sealed in a bag containing Betaplate liquid scintillant (LKB) and were counted in a 1205 Betaplate liquid scintillation counter to determine the associated radioactivity. All samples for each time point were taken in duplicate or triplicate. To obtain values for growth as a percent of control for drug treated cultures, the incorporation of <sup>3</sup>H-thymidine in the presence of drug was divided by the incorporation of

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$^3\text{H}$ -thymidine in the absence of drug and was then multiplied by a factor of 100.

Protein Extracts from Tumor Cells:

Treated or untreated cell lines were washed two to three times with phosphate buffered saline (PBS). Adherent cells were then scraped from the plates in 1 ml of PBS, transferred to a microfuge tube and centrifuged to pellet cells. Non-adherent cultures were first transferred to 15 ml conical tube and centrifuged at 1000 rpm for 5 min. The pellet was resuspended in 5 ml of PBS and centrifuged and the procedure was repeated. This washed pellet was then resuspended in 1 ml of PBS, transferred to a microfuge tube and centrifuged to pellet cells. The resulting cell pellets were resuspended in 50 to 200  $\mu\text{l}$  of 0.2M Tris/0.1M NaCl and were lysed by three cycles of freeze-thawing. Particulate matter was removed by centrifugation and the supernatant was used for creatine kinase enzyme assays, creatine kinase isozyme electrophoresis assays, or to measure total protein levels of cell extracts. Protein content of cellular extracts was determined using the Bio-Rad Protein Assay (Bio-Rad, #500-0006), a dye-binding assay based on the differential change in absorbance of Coomassie Brilliant Blue G-250 from 465 nm to 595 nm in response to binding to protein at various concentrations.

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Creatine Kinase Activity:

Total creatine kinase activity was determined in a coupled reaction using Sigma's creatine kinase kit CK10, where the production of ATP by creatine kinase is coupled to the reduction of nicotinamide adenine dinucleotide phosphate (NADP). The progress of the reaction was monitored spectrophotometrically at 340nm. The coupled reactions are:

CK



HK



G6PD



CK = Creatine kinase

HK = Hexokinase

G6PD = Glucose-6-phosphate dehydrogenase

The coupled reactions were carried out so that the reaction catalyzed by creatine kinase was the rate limiting reaction. Hexokinase and G6PD were in excess. Therefore, the rate of reduction of NADP to NADPH was proportional to creatine kinase activity.

Another test was adopted to assess the proportion of total creatine kinase activity attributable to the brain isoenzyme. The enzyme mixture or cell extract was fractionated by electrophoresis on a 0.8% agarose gel at 90 volts/hr. The gel was then immersed in the

constituents of the coupled reaction described above, with appropriate salts. A colored visible band is generated in the gel at the locations of enzyme activity (Creatine Kinase Kit #715AM, Sigma Chemical Corp.). The migration of each CK isozyme is different, allowing the bands and the activities associated with each isozyme to be distinguished from one another. One can locate the brain isozyme in the gel by running a sample of pure CKBB as a marker.

#### Example 1

##### Level of Creatine Kinase Brain Isozyme Activity in Different Non-Transformed Tumor Cell Lines

Although transformation of cells is associated with alterations in gene expression, very little is known about the cellular processes affected in this transformation process. An important link between the transforming potential of viral oncogene products and the expression of CKB has been demonstrated. To further study the role of creatine kinase in malignancy a panel of transformed and non-transformed cell lines were purchased from the ATCC, were established in culture and were tested for CK expression. Cells were harvested at confluency and were extracted of their protein. To determine total creatine kinase (CK) activity, the protein extracts were assayed spectrophotometrically using coupled reactions (Sigma Kit #49 UV). The

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extracts were also fractionated on 0.8-1.0% agarose gels to separate the CK isoenzymes, and were assayed for CK activity directly in the gel, using Sigma Kit #715 AM. The values determined for total creatine kinase activity, corrected for protein concentration ( $\Delta A_{340}/\text{min}/A_{590} \times 100$ ) for the cell lines listed above are listed in Table 1. It is concluded that many tumor cells in this sampling (lung-, cervical-, and prostate-derived tumor cell lines) expressed a high level creatine kinase, while the non-transformed cells (MRC-5, Vero, BALB/c3T3, and F9) showed a low level of creatine kinase.

The CK isozyme patterns generated in an agarose gel by the coupled reaction and staining procedure described above, were determined for extracts from several tumor cell lines, including small cell lung carcinoma line NCI-H69, prostate carcinoma lines LNCaP.FGC and DU 145, and cervical carcinoma line C-33A. Pure CKBB (brain isozyme), CKMM (muscle isozyme), and CKMB (cardiac isozyme) were run on the gel as controls to indicate the locations of each isozyme in the gel. It was clear from this analysis (data not shown) that brain creatine kinase is the predominant isozyme and thus represents the majority of the creatine kinase activity of these tumor cell lines. These results are consistent with the suggestion that an increased level of expression of the creatine kinase B isozyme is a characteristic alteration in cellular gene expression that occurs during transformation and one that may contribute to the transformed phenotype.

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TABLE 1  
Creatine Kinase Activity in Different  
Tumor Cell Lines

<u>Tissue</u>	<u>Cell Line</u>	<u><math>\Delta A_{340}/\text{min}/A_{590} \times 100</math></u>
Lung	MRC-5	3
	NCI-H69	50
Cervical	C-33A	10
	CaSki	0.5
	SiHa	2
	HeLa	10
	ME180	38
Bone	U-2 OS	1
	Saos-2	2
Kidney	Vero	<1
	293	27
Prostate	PC-3	1
	LNCaP.FGC	30
	DU 145	15
Colon	SW1116	2
	SW403	6
	SW48	3
Embryonal	BALB/c3T3	2
	F9	<1

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Example 2Inhibition of DNA Synthesis in Tumor Cells  
by Inhibitors of Creatine Kinase

The ability of a transformed cell to proliferate indefinitely allows a tumor cell to be cloned and passaged in tissue culture. In vitro, the growth of a population of tumor cells can be quantified through the monitoring of incorporation of  $^3\text{H}$ -thymidine into the DNA of the growing cells. This growth assay creates a convenient means to measure the potential antitumor activity of a compound. Compounds that inhibit the incorporation of  $^3\text{H}$ -thymidine into DNA inhibit DNA synthesis and thereby, inhibit cell proliferation. All such compounds are candidate anticancer drugs. Thus, to determine the potential anticancer activity of inhibitors of creatine kinase, multiple creatine analogs were synthesized and were tested for their ability to inhibit the proliferation of established lines of tumor cells in vitro.

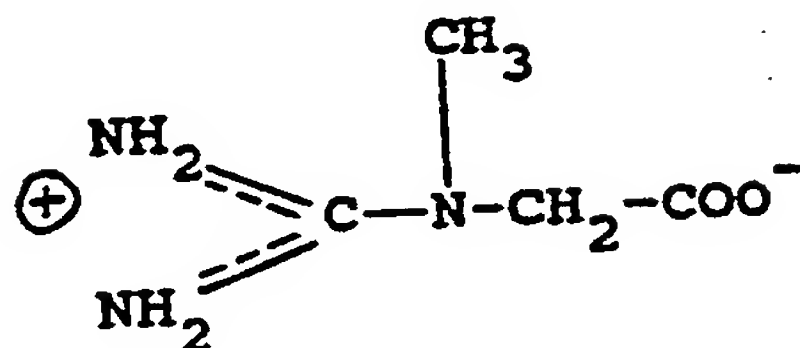
Compounds

Homocyclocreatine (1-carboxyethyl-2-iminoimidazolidine), cyclocreatine (1-carboxymethyl-2-iminoimidazolidine), and 1-carboxymethyl-2-imino-hexahydropyrimidine are cyclic derivatives of creatine. Carbocreatine (3-amino-butyric acid) differs from creatine in that one of the guanidino nitrogens of creatine has been substituted with a

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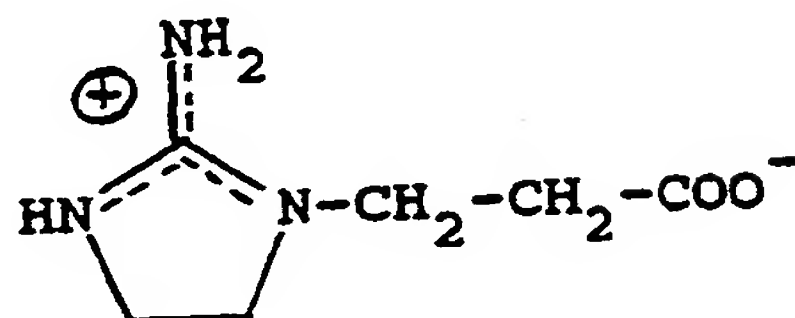
carbon. Guanidino acetate is an analog of creatine that lacks a methyl substituent.

Creatine



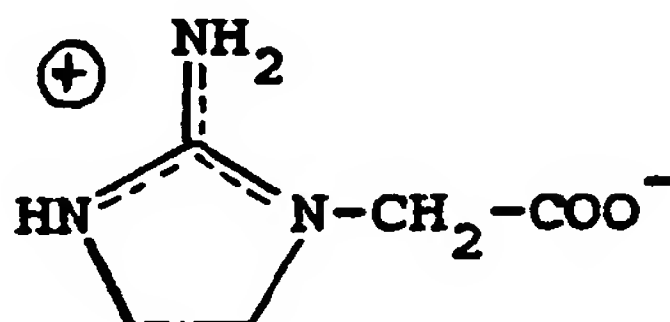
Homocyclocreatine

(1-carboxyethyl-2-iminoimidazolidine)



Homocyclocreatine was shown to be a poor substrate for rabbit muscle creatine kinase, and was reported to react with creatine kinase 10,000-fold more slowly than creatine. In the reverse reaction, at pH 7.0, homocyclocreatine-phosphate (0.2 mM homocyclocreatine-P) was 200,000-fold less active than creatine phosphate as a substrate for rabbit muscle creatine kinase (Roberts, J.J. and J.B. Walker, Arch. Biochem. Biophys. 220: 563-571 (1983)).

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Cyclocreatine (1-carboxymethyl-2-iminoimidazolidine)

Cyclocreatine is similar in structure to homocyclocreatine, differing only by the absence of one methylene group. Cyclocreatine is a better substrate for creatine kinase in vitro than homocyclocreatine, and reacts 1500-fold faster. In the reverse direction, the rate of reaction with creatine kinase in vitro for cyclocreatine-phosphate (cyclocreatine-P) is about 1000-fold faster than that for homocyclocreatine-P (Roberts, J.J. and J.B. Walker, Arch. Biochem. Biophys., 220:563-571 (1983)). McLaughlin et al. reported a relative maximal velocity for reaction of rabbit skeletal muscle creatine kinase with cyclocreatine at 1°C that was 90% that observed with creatine, and a  $K_m$  of 25 mM, only 5-fold higher than that creatine (McLaughlin, A.C. et al., J. Biol. Chem., 247:4382-4388 (1972)). Therefore, under these conditions in vitro, the  $V_{max}/K_m$  ratio for cyclocreatine in the forward reaction is approximately one-fifth that of creatine.

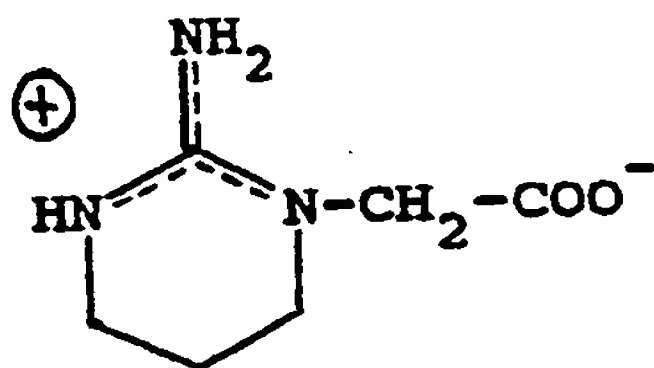
The phosphorylated compound cyclocreatine-phosphate is structurally similar to creatine-phosphate; however, the phosphorous-nitrogen bond of cyclocreatine-phosphate is more stable than that of creatine-phosphate. Cyclocreatine-phosphate

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(3-phosphoryl-1-carboxy-methyl-2-iminoimidazolidine) has a Gibbs free energy of hydrolysis which is approximately 2 kcal/mol below that of creatine phosphate (N-phosphorocreatine) and about 1 kcal/mol above that of ATP. At pH 7 (37°C) in the reverse reaction catalyzed by creatine kinase, the  $V_{max}/K_m$  ratio for cyclocreatine-phosphate is approximately 160-fold lower than for creatine-phosphate (Annesley, T.M. and Walker, J.B., J. Biol. Chem., 253: 8120-8125 (1978); Annesley, T.M. and Walker, Biochem. Biophys. Res. Commun. 74: 185-190 (1977)).

Because cyclocreatine-phosphate binds to the enzyme creatine kinase with a  $K_m$  similar to that of creatine phosphate, yet turns over about 100 times more slowly than does creatine phosphate, cyclocreatine phosphate acts as a competitive inhibitor of creatine kinase. In addition, as cyclocreatine phosphate accumulates in cells, the level of creatine phosphate is suppressed, and hence, a new phosphagen pool is created which is not utilized efficiently in phosphoryl transfer functions.

1-Carboxymethyl-2-iminohexahydropyrimidine (6-CCr)



1-Carboxymethyl-2-iminohexahydropyrimidine (6-CCr) is an analog of creatine, in which two of

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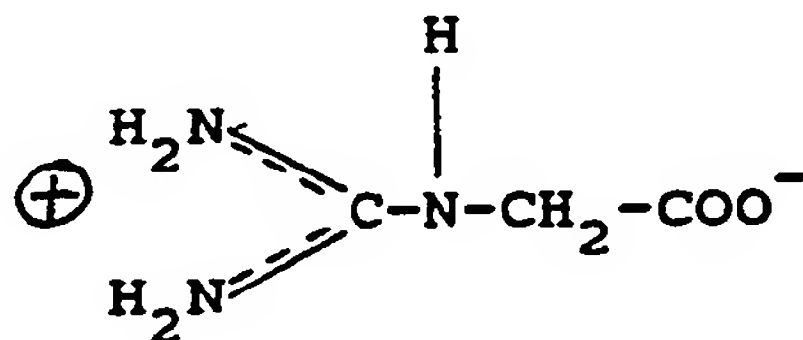


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the nitrogens are joined to form a six-membered ring. 6-CCr differs from cyclocreatine in that the cyclic portion of the molecule has six members instead of five members.

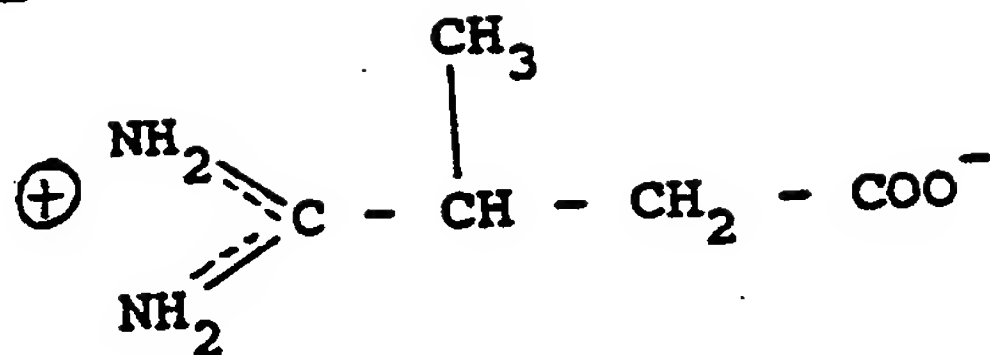
The initial rate of reaction with rabbit muscle creatine kinase of 6-CCr was reported to be 400 times slower than the initial rate of reaction of cyclocreatine (Rowley, G.L. et al., J. Am. Chem. Soc., 93:5542-5551 (1971)). Another group reported no measurable activity of creatine kinase for this compound (McLaughlin, A.C. et al., J. Biol. Chem., 247:4382-4388 (1972)).

#### Guanidinoacetate



Guanidinoacetate is a biosynthetic precursor of creatine and is a moderately active substrate for creatine kinase. McLaughlin and colleagues reported a maximal velocity of reaction with rabbit muscle creatine kinase 10% that of creatine, and a  $K_m$  about 14-fold below that for creatine (McLaughlin, A.C. et al., J. Biol. Chem., 247: 4382-4388 (1972)). Thus, while guanidinoacetate is a better substrate for the enzyme than homocyclocreatine or 6-CCr, it is a poorer substrate than cyclocreatine in the forward reaction.

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Carbocreatine

Carbocreatine is an analog of creatine in which one of the guanidino nitrogens is replaced with a carbon. Nguyen reported that the initial rate of the reaction with rabbit muscle creatine kinase and carbocreatine was less than 1% of that of creatine. Carbocreatine-phosphate was shown to be more stable than creatine-phosphate in vitro (Nguyen, Ann Cae Khue, Ph.D. Thesis in Pharmaceutical Chemistry, University of California, San Francisco, CA, 1983).

Homocyclocreatine Inhibits the Proliferation of DU 145 Cells in a Dose-Dependent Manner

The results of an experiment illustrating the antiproliferative effect of the homocyclocreatine preparations is shown in Figure 8. In this particular experiment, the effect of a homocyclocreatine preparation on the DNA synthesis of the DU 145 cell line, derived from a prostatic carcinoma which expresses a high level of creatine kinase BB, was determined. The concentration of drug was varied from 0 mM to 10 mM.

DNA synthesis was monitored under each regimen by measurement of incorporation of  $^3\text{H}$ -thymidine once a day for 5 days. Each day after initial drug addition, the media was changed and the drug was

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replenished. Control cells received new media with no drug addition. Figure 8 shows a plot of the incorporation of  $^3\text{H}$ -thymidine as percent of the untreated control versus the concentration of homocyclocreatine for 1, 2, 3, 4 or 5 days of exposure to the drug. It is clear that there is both a dose- and time-related response to homocyclocreatine. As the concentration of or duration of exposure to homocyclocreatine increased, the % growth of DU 145 cells decreased.

In addition, from these curves, the ID50 or the concentration at which 50% inhibition of growth is reached for each period of treatment (1-5 days) can be determined. The values for the ID50 range from 3.0 mM homocyclocreatine for one day of treatment to 0.7 mM homocyclocreatine for five days of treatment. Thus 50% inhibition of growth of prostate tumor cell line DU 145 was achieved by exposure to approximately 1.0 mM homocyclocreatine for a period of five days.

#### Effects of Creatine Kinase Inhibitors on DNA Synthesis of Normal and Multiple Tumor Cell Lines

Given the dose-related response observed for homocyclocreatine against DU 145 cells, homocyclocreatine preparations and other inhibitors of creatine kinase were assayed for antiproliferative activity against a panel of cell lines. Eighteen different cell lines were treated with homocyclocreatine (HcCr), cyclocreatine (CCr), and 1-carboxymethyl-2-imino-hexahydropyrimidine (6-CCr) and guanidinoacetate. The cell lines used

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in these experiments included the non-transformed lung fibroblast MRC-5 cell line, the immortal mouse embryo cell line BALB/c3T3, four human cervical carcinoma cell lines (C-33A, CaSki, SiHa, and HeLa), two human osteogenic sarcomas (U-2 OS and Saos-2), a non-transformed African Green Monkey kidney cell line (Vero), an adenovirus (type 5) transformed human primary embryonal kidney cell line (293), three human prostate carcinoma cell lines (PC-3, LNCaP.FGC, and DU 145), three human colon adenocarcinomas (SW1116, SW403, and SW48), a human small cell lung carcinoma cell line (NCI-H69), and the F9 mouse embryonal carcinoma cell line.

In the first set of experiments, the cell lines were treated with 0, 1, 2, or 4 mg/ml drug for five days, and were assayed for growth by incorporation of <sup>3</sup>H-thymidine. In the second set of experiments, the same eighteen cell lines were treated with 4 mg/ml drug for five days before assaying for growth.

The data from both sets of experiments are summarized in Table 2. In some cases, values from a third experiment, using a similar protocol, are included in the table (in parentheses). For each of the eighteen cell lines in Table 2, the inhibition of growth by each drug at 4 mg/ml is recorded as a percent of the untreated control (4 mg/ml = 24 mM for each drug).

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TABLE 2

Effect of Drug on <sup>3</sup>H-Thymidine Incorporation  
(Incorporation as a % of Untreated Control)

Tissue	Tumor Type	(CK activity)	Cell Line	#236 HcCr	#216 6-CCr	#229 CCr	#237 GA
Lung	Non-transformed (Human)	3	MRC-5	102 84 (112)	116 118 (114)	32 24	75 --
	Small Cell Carcinoma (Human)	50	NCI-H69	28	29	35(1mg/ml)	81
Cervical	Carcinoma (Human)	10	C-33A	32 63	40 68	17 26	374 --
	Epidermoid Carcinoma (Human)	0.5	CaSkI	9 5 (4)	25 5 (2)	33 68	86 --
	Squamous Carcinoma (Human)	2	SiHa	56 92	48 83	7 20	131 --
	Epitheloid Carcinoma (Human)	10	HeLa	83 102	60 65	33 37	89 --
Bone	Osteogenic Sarcoma (Human)	1	U-2 OS	101 0.06	55 10	69 0.02	147 --
	Osteogenic Sarcoma (Human)	2	Saos-2	64 0.03	825 27	7 0.02	49 --
Kidney	Normal Kidney (African Green Monkey)	1	Vero	112 120	117 119	-- 79	97 --

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TABLE 2 (cont)

Effect of Drug on <sup>3</sup>H-Thymidine Incorporation  
(Incorporation as a % of Untreated Control)

Tissue	Tumor Type	(CK activity)	Cell Line	#236 HCC	#216 6-CCR	#229 CCR	#237 GA
Prostate	Ad 5 Transformed Embryonal (Human)	27	293	9	19	--	63
				4	2	11	--
Prostate	Adenocarcinoma (Human)	1	PC-3	109	88	34	93
				82	87	26	--
Colon	Adenocarcinoma, Lymph node metastasis (Human)	30	LNCaP.FGC	85	14	0.05	54
				0.04	13	0.03	--
Colon	Carcinoma, brain metastasis (Human)	15	DU 145	7	12	24	108
				0.03	5	0.02	--
Colon	Adenocarcinoma (Human)	2	SW1116	74	80	0.34	5
				0.04	102	0.14	--
Colon	Adenocarcinoma (Human)	6	SW403	1	91	0.12	9
				0.47	0.5	0.17	--
Embryonal	Adenocarcinoma (Human)	3	SW48	63	84	0.64	4
				72	74	12	--
Embryonal	Embryo (Mouse)	2	BALB/c3T3	3	11	110	4
				6 (9)	7 (39)	59	--
Embryonal	Embryonal carcinoma (Mouse)	1	F9	11	21	57(1mg/ml)	204
				217	431	7	--

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In some cases, the percent inhibition of growth of a cell line by a given drug appears to vary significantly. Variations in the culture conditions may explain these fluctuations. For example, in the second experiment with the Saos-2 osteogenic sarcoma line, the cells appeared to be dying independent of the administration of drug. The LNCaP.FGC prostate cells tended to grow in clumps and become nonadherent, leading to inconsistency in harvesting of the cells. Further, because healthy LNCaP.FGC cells are somewhat nonadherent, it is difficult to visually identify generally unhealthy cells and distinguish these from cells actually inhibited by drug. The F9 teratocarcinoma cells also show variation, possibly due to fluctuations in pH or to the fact that the cells are grown on a gelatin matrix and detach from the substratum easily. The incorporation of <sup>3</sup>H-thymidine by the untreated F9 cells was inconsistent. Lastly, some of the fluctuation in values for the colon cell lines may result from acidification of the medium, which is not buffered by sodium bicarbonate.

The values are quite consistent in many cases. Furthermore, by conducting multiple experiments one can identify the effect of drug apart from artifactual fluctuations. For example, homocyclocreatine and 6-CCr reproducibly inhibited the growth of DU 145. Similarly, cyclocreatine strongly and reproducibly inhibited the growth of the cervical carcinoma line ME-180 (see below) as well as all a series of colon carcinoma cell lines.

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An example of the dose-response of colon carcinoma cell lines to cyclocreatine is shown in Figure 9. The effect of cyclocreatine on five colon carcinoma cell lines obtained from the ATCC (SW1116, ATCC CCL 233; SW48, ATCC CCL 231; SW620, ATCC CCL 227; Caco-2, ATCC HTB 37 (CAC02); and WiDr, ATCC CCL 218, (WIDR)) was determined. In this case, each cell line was incubated for 7 days in the presence of 0.0 mM, 3.5 mM, 7.0 mM, 14.0 mM, 28.0 mM, 42.0 mM, 56.0 mM or 70.0 mM cyclocreatine. Figure 9 shows a histogram illustrating incorporation of  $^3\text{H}$ -thymidine (CPM) versus the concentration of cyclocreatine for each cell line. As the concentration of cyclocreatine increases, the incorporation of  $^3\text{H}$ -thymidine into the DNA of each cell line decreases. This clear dose-related response establishes the anti-proliferative effect of cyclocreatine against these colon cell lines.

From these examples and from the results of many other experiments, it is clear that cyclocreatine and the homocyclocreatine and 1-carboxymethyl-2-imino-hexahydropyrimidine preparations can inhibit the growth of a variety of tumor cell lines in vitro. In contrast, none of the compounds has a strong or reproducible effect against the non-transformed cell line Vero. Similarly, the non-transformed lung fibroblast MRC-5 is unaffected by HcCr and 6-CCr and weakly affected by cyclocreatine, suggesting that normal tissues may tolerate homocyclocreatine, 6-CCr, or cyclocreatine at doses not tolerated by tumor tissue.

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### Trends of Anti-Proliferative Activity of Creatine Kinase Inhibitors

The comparison of results from multiple experiments such as those described above reveals several trends. Figure 10 summarizes the results of a series of proliferation assays using a representative value from one such experiment for each of 15 of the cell lines tested above and one additional cell line, ME-180, which is derived from a cervical carcinoma. In Figures 10A-10D, the height of the bars represents the incorporation of <sup>3</sup>H-thymidine as a percent of the corresponding untreated control for cells treated with 4 mg/ml of a homocyclocreatine preparation (Figure 10A, HcCr), 4 mg/ml of a 1-carboxymethyl-2-iminohexahydropyrimidine preparation (Figure 10B, 6-CCr), 4 mg/ml cyclocreatine (Figure 10C), or 4 mg/ml guanidinoacetate (Figure 10D, GA). The trends of anti-proliferative activity highlighted by these figures is summarized below.

First, the HcCr and 6-CCr preparations showed a nearly identical pattern of specificity, such that lines that were strongly affected by the former were strongly affected by the latter. Similarly, the lines that were marginally affected by one drug were only marginally affected by the other. Although the HcCr, 6-CCr, and cyclocreatine preparations all had a minimal effect on DNA synthesis of non-transformed cells, cyclocreatine showed a different spectrum of activity than the HcCr and 6-CCr preparations against the panel of cell lines.

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The sensitivity of colon cell lines to inhibition by cyclocreatine documented in Figure 9 is evident in Figure 10C as well. Another trend of particular interest is emphasized in Figure 11. Figure 11 shows the growth as a percent of control for five cervical lines treated with HcCr, 6-CCr or CCr. The C-33A, HeLa and SiHa lines are derived from primary tumors, while the CaSki and ME-180 lines are derived from metastases. The HcCr and 6-CCr preparations showed preferential inhibition of the cervical metastatic cell lines relative to the non-metastatic lines. In contrast, CCr did not show this preference for metastatic cell types. Note that the HcCr and 6-CCr preparations also tended to inhibit the growth of the metastatic prostate lines DU 145 (brain metastasis) and LNCaP.FGC (lymph node metastasis), although the results for the LNCaP.FGC line were less reproducible. Again, the non-metastatic prostate line, PC-3, was not strongly inhibited by the HcCr or 6-CCr preparations.

The metabolism of metastatic cells may differ from that of non-metastatic cells. Thus, the metastatic lines may be more susceptible to inhibition of a function that is essential, though not necessarily unique, to those cells. In addition, the function or functions inhibited in the metastatic lines may also be necessary to or involved in the process of metastasis. The HcCr and 6-CCr preparations could be affecting the processes that control, activate, or are required for metastasis, either directly or indirectly. The

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drugs could be administered alone or in combination with other drugs or therapies to prevent metastasis. They may also be particularly effective in killing tumor cells or reducing the growth of tumors in aggressive or late stage disease (especially in prostatic and cervical disease), where metastasis has already taken place.

Usually, late stage prostatic cancers are unresponsive to hormone therapy. In fact, the DU 145 cell line does not respond to hormone therapy. Thus, drugs which inhibit growth of the DU 145 cell line, such as the homocyclocreatine and 1-carboxymethyl-2-imino-hexahydropyrimidine preparations could provide effective therapies for these untreatable tumors. Identification of the site of action of the drugs may shed light on the processes of metastasis and may suggest other methods of interfering with metastasis.

It is important to emphasize that the MRC-5 and Vero cell lines are largely unaffected by the HcCr and 6-CCr preparations. The MRC-5 and Vero lines are tissue culture versions of "normal" cell lines. Thus, HcCr and 6-CCr may also spare normal cells, suggesting they may have low toxicity in an individual.

Another important trend observed in multiple experiments is that cyclocreatine inhibited the growth most consistently of tumor cells expressing the highest levels of creatine kinase. For example, ME-180, 293, and LNCaP.FGC cells, all expressing very high levels of creatine kinase (see Table 1),

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are strongly affected by cyclocreatine. For HcCr and 6-CCr on the other hand, no such obvious correlation between creatine kinase activity and growth inhibition is seen.

The precise mode and site of action for each of these three preparations (CCr, HcCr, 6-CCr) is not known. However, it is noted in light of the different specificity (i.e., different pattern of cell lines affected) of the HcCr and 6-CCr preparations on the one hand, and that of the cyclocreatine preparation on the other hand, that HcCr and 6-CCr are distinct from cyclocreatine in that they are very poor substrates for creatine kinase in the forward reaction and are thus phosphorylated very slowly. It is therefore possible that the two classes of compounds are acting through a different mechanism.

In view of this possibility, it is interesting to note that the HcCr and 6-CCr preparations do not retain full anti-proliferative activity upon multiple recrystallizations of the major product of the referenced synthetic protocols, while cyclocreatine does retain complete activity through multiple recrystallizations of the major product. Although it is most probable that cyclocreatine is the active component of its preparation, it is possible that the active component of the HcCr and 6-CCr preparations is not the expected creatine analog. The cited literature preparations for HcCr and 6-CCr do lead reproducibly, however, to the synthesis of the anti-proliferative activity

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illustrated here. Three preparations of HcCr and two preparations of 6-HcCr have produced similar patterns of activity. Therefore, as used herein, "homocyclocreatine" and "1-carboxymethyl-2-imino-hexahydropyrimidine" are used to refer to preparations containing homocyclocreatine and 1-carboxymethyl-2-imino-hexahydropyrimidine, respectively, the preparations having the activity exemplified and claimed herein and which were obtained from the referenced source or made by the synthetic methods referenced herein.

Guanidinoacetate showed a third pattern of inhibition against the panel of cell lines. In the case of guanidinoacetate, two series of experiments to date have shown strong inhibition of the growth of all three colon lines and of the BALB/c3T3 line. The remainder of the lines were largely unaffected by the drug. Again, the mode of action of guanidinoacetate is not known. Because changes in the pH of the medium under the culture conditions were not quantified, data for the colon cell lines should be interpreted cautiously.

Carbocreatine, tested in similar experiments, inhibited the growth of both transformed and non-transformed cell lines tested to approximately the same extent (data not shown). The calculated ID50 of carbocreatine for these lines was about 1 mM. In addition there was no correlation of the anti-proliferative effect of the compound with the creatine kinase activity of cell line being tested. These results suggest that this drug may not have

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the desired specificity for tumor cells and may not be functioning through creatine kinase.

The differences between cell lines in susceptibility to a given drug may reflect differences in the cause or severity of transformation or other properties of the cell lines, for example. The tendency of the four drugs (HcCr, 6-CCr, CCr and GA) to inhibit a particular subset of lines, in essence displaying selective killing, may also result in reduced toxicity in vivo.

### Example 3

#### Guanidino Compounds with Little or No Effect on Tumor Cell Growth

Ten additional guanidino (i.e., creatine and analogs) compounds were tested for their ability to inhibit growth of a number of cell lines. The structures of the ten compounds tested, references, and sources of purchase are listed in Table 4. In these experiments, the cell lines were exposed to a drug at concentrations from 1 mg/ml to 4 mg/ml for a period of five days. After five days, cells were washed to remove the drug, and growth was assayed by incorporation of <sup>3</sup>H-thymidine into DNA.

At a concentration of 1 mg/ml, after five days of exposure to drug and under the conditions of the assay, these ten guanidino compounds showed little or no effect on the growth of the cell lines. In most cases, only modest effects on cell growth were

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observed with the drugs at a concentration of 4 mg/ml (Table 5). In addition,  $\beta$ -guanidino-propionic acid and N-methyl-hydantoic acid inhibited the growth of the non-transformed MRC-5 cell line more strongly than they inhibited growth of many of the transformed cell lines, suggesting that these drugs may not have the desired specificity for tumor cells.

Although colon cell lines SW1116 and SW48 appeared to be strongly inhibited by N-methyl-hydantoic acid and 1-carboxymethyl-2-iminoimidazolidine-4-one, the control cells appeared to be unhealthy. (As noted above, the colon cell lines are difficult to maintain in tissue culture due to the sensitivity of the cells and possible acidification of the medium.) The asterisked values in Table 5 should be interpreted with caution, because the counts per minute of  $^3\text{H}$ -thymidine incorporated in the control samples were quite low, these values may not be significant.

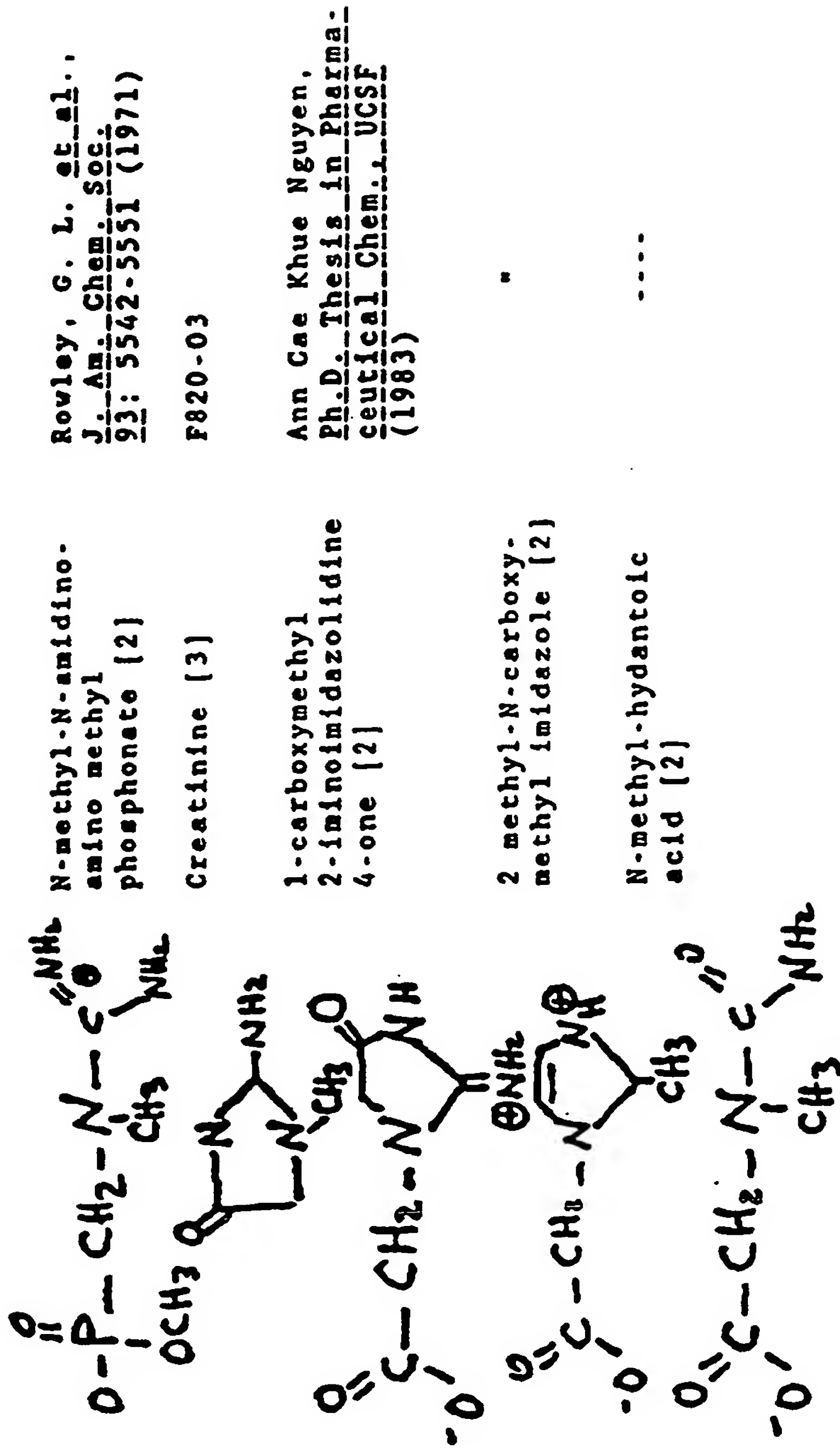
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TABLE 4

GUANIDINO COMPOUNDS (CREATINE AND ANALOGUES)  
DEMONSTRATING LITTLE OR NO EFFECT ON TUMOR  
GROWTH UNDER CONDITIONS USED

Structure	Compound Name	Reference
	$\beta$ -Guanidino-propionic acid [1]	G6878
	N-methyl amidino-methyl glycine [2]	Rowley, G. L. et al., J. Am. Chem. Soc. 93: 5542-5551 (1971)
	N-ethyl-N-amidino glycine (N-ethyl guanidino acetate) [2]	Richmond, J. J. & Walker, J. Acc. Biochem. Biophys. 252: 564-570 (1982)
	N-acetimidoyl-sarcosine [2]	Wang, T., J. Org. Chem. 39: 3591-3594 (1974)
	N-propyl-N-amidino glycine (N-propyl guanidino acetate) [2]	Rowley, G. L. et al., J. Am. Chem. Soc. 93: 5542-5551 (1971)

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[1] Sigma  
[2] Dalton  
[3] Baker

Rowley, G. L. et al.,  
J. Am. Chem. Soc.  
93: 5542-5551 (1971)

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Ann Gae Khue Nguyen,  
Ph.D. Thesis in Pharma-  
ceutical Chem., UCSF  
(1983)

N-methyl-N-amidino-  
amino methyl  
phosphonate [2]

Creatinine [3]

1-carboxymethyl  
2-iminoimidazolidine  
4-one [2]

2-methyl-N-carboxy-  
methyl imidazole [2]

N-methyl-hydantoic  
acid [2]

TABLE 5  
Effect of Drug on Incorporation of <sup>3</sup>H-Thymidine  
(% of Untreated Control)

Amira #	209	210	211	213	260	274	259	261	262	276
Exp #	42	42	42	42	81	81	75	75	75	84
Compound	A	B	C	D	E	F	G	H	I	J
Cell Line										
293	78	91	85	77	97	166	185	115	223	209
Vero	75	83	166	77	74	117(2mg/mL)	80	88	73	125
Balb-c 3T3	106	115	130	120						
C-33A	105	111	106	96			126	116	133	27
CaSki	102	106	108	76	45	66	88	97	89	
SiHa	95	91	108	91						
HeLa	107	128	112	169			130	148	107	94
DU 145	116	111	128	77	121	168	95	80	86	
PC3	111	100	119	69						
LnCAP	71	76	77	36						
SAOS-2	98	90	80	68						
U2OS	100	101	142	112						
SW1116	141	0	158	0			120	150	50	
SW48	128	0	131	0			172*	199*	99*	
SW403										
MRC5	103	53	37	34			113	91	180*	
H69	92	82	98	87						

Key:  
A = 2-methyl-N-carboxymethylimidazole  
B = 1-carboxymethyl-2-iminoimidazolidine-4-one  
C = Beta-guandinopropionic acid  
D = N-methyl-hydantoic acid  
E = N-methylamidino-N-methylglycine  
F = N-methyl-N-amidino amino methyl phosphonate  
G = N-ethyl-N-amidinoglycine  
H = N-acetimidoylsarcosine  
I = N-propyl-N-amidinoglycine  
J = creatinine



Example 4Inhibition of Colony Formation of Established Tumor  
Cell Lines in Soft Agar by Inhibitors  
of Creatine Kinase

The human tumor colony assay (or stem cell assay) is an in vitro culture system that differs from the DNA synthesis assay by the use of a semisolid medium support. Only transformed cells capable of self-renewal grow in this support system. Chemosensitivity testing of specific agents in the colony assay has revealed a definite heterogeneity in drug sensitivity of different tumors, and clinical correlations have now been made between chemosensitivity to an agent in this assay and the response of patients to the corresponding agent in chemotherapy. In light of the predictive value of this assay, the effect of inhibitors of creatine kinase on the ability of transformed cells to form colonies in soft agar was tested.

Tumor Cells

Human tumor cells were derived from established lines of tumor cells. Cells were purchased from the American Type Culture Collection (ATCC), Rockville, MD, except for the A2058 cell line, which was received from the Dr. M. Beckner, U.S. National Cancer Institute. Since purchase, cells have been passaged twice weekly at a subcultivation ratio of 1:6 or 1:8. Passage of adherent cells entailed

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removal of growth medium, incubation of monolayers in 3 mls of 0.25% trypsin (J.R.H. Biosciences) for several minutes until cells detached, followed by addition of 20 mls of fresh growth medium to inactivate the trypsin.

For soft agar colony assays, cells were collected from 75 cm<sup>2</sup> flasks by dissociation in 5 mls of 0.25% trypsin (J.R.H. Biosciences). Trypsin was inactivated with 10 mls of EMEM + 10% FBS. Cells were pelleted in a Beckman table top centrifuge for 5 mins at 1500 rpms. The supernatant medium was removed by aspiration and the pellet was resuspended in 1-5 mls of IMDM Enriched Media (see below). Cells harvested by trypsinization were counted in a hemocytometer to determine the number of viable cells. From this stock, generally from  $1.5 \times 10^3$  to  $7.5 \times 10^3$  cells were added to each 35 mm dish.

#### Media for Cell Line Maintenance

For growth of stock tumor cell lines ME-180 (ATCC HTB 33), DU 145 (ATCC HTB 81), SK-N-MC (ATCC HTB 10; (SKNMC)), and U-87 MG (ATCC HTB 14) cells, medium consisted of 90% Minimum Essential Medium (Eagle's with Earle's Balanced Salts, without L-glutamine, J.R.H. Biosciences 51-412, EMEM), 10% fetal bovine serum, 100 U/ml Pen/Strep, 2 mM L-glutamine, 1 mM Na pyruvate. (J.R.H. Biosciences). Cells were grown in 75 cm<sup>2</sup> flasks at 5% CO<sub>2</sub>, 37°C.

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For SW48 (ATCC CCL 231) cells, growth medium consisted of 90% L-15 (Leibovitz) Medium (with L-glutamine, J.R.H. Biosciences 51-201), 10% fetal bovine serum, 100 U/ml Pen/Strep, plus 2 mM L-glutamine, 1 mM Na pyruvate. Cells were grown in closed 75 cm<sup>2</sup> flasks at 37°C.

For CaSki (ATCC CRL 1550) and NCI-H69 (ATCC HTB 119; (H69)) cell lines, medium consisted of 90% RPMI 1640 Medium (without L-glutamine, JRH Biosciences 51-502), 10% fetal bovine serum, 100 U/ml Pen/Strep, plus 2 mM L-glutamine, 1 mM Na pyruvate (J.R.H.). Cells were grown in 75 cm<sup>2</sup> flasks at 5% CO<sub>2</sub>, 37°C.

For NIH:OVCAR (ATCC HTB 161, (OVCAR)) colon adenocarcinoma cells, medium consisted of 90% RPMI 1640 Medium (without L-glutamine, JRH Biosciences 51-502), 10% fetal bovine serum, 100 U/ml Pen/Strep, plus 2 mM L-glutamine, 1 mM Na pyruvate (J.R.H.), 10 ug/ml insulin (Sigma). Cells were grown in 75 cm<sup>2</sup> flasks at 5% CO<sub>2</sub>, 37°C.

For A2058 human metastatic melanoma cells, medium consisted of 90% Dulbecco's Modified Eagle's Medium (with 4500 mg/L Glucose, without L-glutamine, J.R.H. Biosciences 51-432), 10% fetal bovine serum, 100 U/ml Pen/Strep, plus 2 mM L-glutamine, 1 mM Na pyruvate (J.R.H.). Cells were grown in 75 cm<sup>2</sup> flasks at 5% CO<sub>2</sub>, 37°C.

#### Medium for Colony Assays

For the colony assays, all cells were incubated in IMDM Enriched Medium, which consisted of 77% Iscove's Modified Dulbecco's Medium (IMDM), 2 mM

L-glutamine, 4 mM  $\text{CaCl}_2$ , 2300 mg/l  $\text{NaCl}_2$ , 3 U/ml insulin, 0.5 mg/ml DEAE Dextran, 1.5% BSA (Sigma A-9418), 10% FBS (Fetal Bovine Serum), 10% HS (Horse Serum), 2 mM Na Pyruvate, 100 U/ml Pen/Strep. To prepare IMDM Enriched Medium all ingredients except BSA were mixed in a 1000 ml beaker with a stir bar. BSA was then added to the mixture and allowed to dissolve for several hours. The medium was then filtered with a 0.2  $\mu\text{m}$  filter and was divided into 250 ml aliquots and stored frozen at  $-20^\circ\text{C}$ .

#### Preparation of Drug Stocks and Treatment Protocols

For each experiment, a freshly prepared stock concentration of 16 mg/ml cyclocreatine was made in IMDM Enriched Media. For measuring the clonogenicity of cells during continuous exposure to drug, an aliquot of the stock solution of cyclocreatine was added to the cells and agar immediately before plating to achieve a final concentration of cyclocreatine between 0 and 8.0 mg/ml. In general, there were six doses of drug per experiment and each dose was performed in quadruplicate: four 35 mm dishes for each drug concentration, including the vehicle-treated control.

For measuring the clonogenicity of cells after a limited exposure to drug, the cells were pretreated with drug for 48 hours before being plated in soft agar without drug:  $1 \times 10^5$  cells were plated per 25  $\text{cm}^2$  flask and were allowed to adhere (at least 6 hours). The growth media was then

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aspirated and replaced with 5 mls of media containing cyclocreatine at 0, 0.5, 1.0, 2.0, 4.0 or 8.0 mg/ml. Typically there were two 25 cm<sup>2</sup> flasks per drug concentration. The cells were exposed to the drug for 48 hours and then were removed from the flask with 2 mls of 0.25% trypsin. To remove residue of the drug, the 2 ml cell suspension was added to a centrifuge tube containing 5 ml of IMDM Enriched Medium and was centrifuged for 5 min at 1500 rpm. The cell pellet was resuspended in fresh IMDM Enriched media. The cells were then counted and added to agar without drug. The same number of viable cells were plated from each drug dose. In these cases, colony formation is assayed in the absence of drug. This assay measures the ability of the cells remaining viable after the limited exposure to drug to form colonies. The number of colonies formed does not reflect the loss of cells during the limited exposure to the drug.

#### Plating in Soft Agar

The soft agar used in the assays consisted of two layers: a base feeder layer of 0.5% agar and medium and a less solid top layer (0.3% agar), which holds the tumor cells. To form the base layer, approximately 1.5 mls of 0.5% Bacto Agar containing medium and serum was pipetted into 35 mm dishes and allowed to solidify at room temperature for 5 min: a 2% Bacto Agar (Difco) Solution in 1X PBS (JRH Biosciences) was sterilized by autoclaving and was stored at 4°C when not in use. Solidified agar

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stored at 4°C was first boiled until liquid (about 5 minutes) and was then cooled to 42°C. An aliquot was checked for proper temperature with a thermometer. Three 35 mm petri dishes were placed inside a 100 mm petri dish. One of the plates was placed into the larger plate and was filled with water as needed in order to prevent dehydration of the agar in the course of the experiment. Two of the plates had lids, and were used for addition of the agar, cells and drug. From the 2% Bacto Agar solution, a second solution of 0.5% Bacto Agar was prepared using pre-warmed (37°C) 80% IMDM, 20% fetal bovine serum, 100 U/ml Pen/Strep, 2 mM L-glutamine, 1 mM Na pyruvate as the diluent.

The top layer, consisting of agar, medium, serum, cells and, in some cases, drug, was prepared in a 15 ml tube. Drug (0 to 1.8 ml) and cells (10  $\mu$ l to 0.3 ml) were mixed with IMDM Enriched Media (an amount necessary to bring volume to 2.5 ml) followed by the addition of 0.5 mls of 2% Bacto Agar kept at 42°C (final agar concentration = 0.3%; total volume = 3 mls). This cocktail was then mixed with a 5 ml pipette. Approximately 1.2 mls of this mixture was pipetted into each of two 35 mm dishes containing a solidified layer of 0.5% Bacto Agar placed within the larger 100 mm plate. For each treatment group this process was repeated, until there were a total of four 35 mm dishes per treatment group. Approximately 3 mls of sterile water was placed in the remaining, open 35 mm petri dish within the 100 mm plate. These plates were

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left at room temperature for about 5 minutes to allow the agar to set and were then incubated at 37°C, 5% CO<sub>2</sub> for 3 weeks.

After 21 days, generally, the colonies were stained with a vital stain to determine viability and to aid visualization. 0.5 mls (0.5 mg/ml in water) of the P-iodonitrotetralium violet (Sigma) was added dropwise over the agar. The dye is an electron acceptor of multiple cellular dehydrogenases. After addition of the dye, the plates were returned to the incubator at 37°C, 5% CO<sub>2</sub> for 24 hrs. Viable cells were stained a bluish-purple color. Stained colonies consisting of greater than 50 cells were identified at 40X magnification with a phase contrast microscope.

#### Effect of Continuous Exposure to Cyclocreatine

The in vitro chemosensitivity of tumor cells to continuous exposure to cyclocreatine was measured using the double-layer soft agar assay described above. Single cell suspensions ( $1.5 \times 10^3$  to  $7.5 \times 10^3$  cells per 35 mm dish) from different established human tumor cell lines were plated separately in soft agar, typically in the presence of one of at least six different concentrations of cyclocreatine (between 0.25 and 8.0 mg/ml). Untreated control cells were plated with vehicle only. Each treatment and control group was performed in quadruplicate (four 35 mm dishes per group). After 21 to 37 days, colonies that had formed were counted under 40X magnification with a phase contrast microscope. (In

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many cases the colonies were stained with a vital stain to facilitate counting.) The number of colonies formed in the untreated control groups were between approximately 50 to 750 colonies. (Experiments with less than an average of 48 colonies per control plate are not included.) The mean number of colonies formed in each treatment and control group was plotted to determine the dose required to inhibit plating efficiency by 50%.

The plating efficiency of H69 (small cell lung carcinoma), ME-180 (cervical carcinoma), SKNMC (neuroblastoma metastasis), and SW48 (colon adenocarcinoma) cells was severely reduced by continuous exposure to high doses of cyclocreatine. Clonal growth of each of these cell types was inhibited to 50% of control values by continuous exposure to approximately 0.5 mg/ml (3.5 mM) cyclocreatine. The inhibitory effect of cyclocreatine on the clonogenicity of these cells is tabulated below and is shown in each instance to be dose-related. That is, the higher the drug dose, the fewer number of colonies were formed. For H69 cells, at continuous exposure to 2 mg/ml cyclocreatine, no colonies of greater than 50 cells had formed within 23 days. For ME-180 cells, clonal growth was inhibited by 99% at 2 mg/ml cyclocreatine. For SKNMC cells, clonogenicity was reduced by 98% at 2.0 mg/ml cyclocreatine as compared with the untreated control. Growth of SW48 cells was reduced by 95% at 1.0 mg/ml cyclocreatine

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as compared with the untreated control. No colonies of the minimum size formed at either 1.5 mg/ml or 2.0 mg/ml cyclocreatine.

Table 6

<u>Ccr (mg/mL)</u>	<u>Average Number of Colonies Formed</u>			
<u>(Cell Line)</u>	<u>H69</u>	<u>ME-180</u>	<u>SKNMC</u>	<u>SW48</u>
0.0	45	110	113	334
0.25	n.d.*	n.d.	n.d.	252
0.5	28	39	75	157
0.75	n.d.	n.d.	n.d.	41
1.0	12	17	28	18
1.5	6	3.5	n.d.	0
2.0	0	1	2	0
2.5	0	1	n.d.	n.d.
3.0	0	1	n.d.	n.d.
4.0	0	0	0	n.d.
8.0	0	0	0	n.d.

\* n.d. indicates not done.

The plating efficiency of OVCAR cells was reduced significantly, although not as severely as for the cell lines shown in Table 6. The CD50 for OVCAR cells was 0.8 mg/ml. However, the CD50 may be somewhat overestimated for this cell line, because the average number of colonies in the control plates exceeded 1000, making an accurate count difficult.

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At 2.0 mg/ml, 4.0 mg/ml, and 8.0 mg/ml cyclocreatine, clonal growth of OVCAR cells was reduced by 89%, 93%, and 95%, respectively, as compared with the untreated control.

The plating efficiency of U-87 MG (glioblastoma, astrocytoma) cells was reduced by 50% at a 10X higher dose than that of ME-180 cells: 5.5 mg/ml (39 mM). At 4.0 mg/ml, colonies were reduced by 31%, and at 8.0 mg/ml, colonies were reduced by 80%, relative to the untreated control.

Under the conditions of the assay, the growth of A2058 cells was unaffected by the highest concentration of cyclocreatine tested, which was 8.0 mg/ml or 56 mM cyclocreatine.

#### Effect of 48 Hour Exposure of Cells to Cyclocreatine

The in vitro chemosensitivity of tumor cells to a 48 hour exposure to cyclocreatine was also measured using the double-layer soft agar assay.  $1 \times 10^5$  cells from several different cell lines were plated in 25 cm<sup>2</sup> flasks and treated for 48 hours with selected concentrations of cyclocreatine in duplicate before being plated in soft agar in the absence of drug. Untreated control cells were incubated for an equivalent time with vehicle only. Single cell suspensions ( $1.5 \times 10^3$  to  $7.5 \times 10^3$  cells per 35 mm dish) from each flask were plated in the absence of drug in two 35 mm dishes. Each treatment and control group was thus performed in quadruplicate (two 25 cm<sup>2</sup> flasks into four 35 mm dishes per group). After 21 to 37 days, colonies

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that had formed were counted under 40X power with a phase contrast microscope. In many cases the colonies were stained with a vital stain to facilitate counting. Approximately 50 to 750 colonies were formed in the untreated control groups. (Experiments with less than an average of 48 colonies per control plate are not included.) The mean number of colonies formed in each treatment and control group was plotted to determine the dose required to inhibit plating efficiency by 50% (CD50).

The CD50 values for 48 hour exposure to cyclocreatine for each of the five cell lines tested is tabulated below (Table 7).

Table 7

Cell Line	CD50 (48 hour exposure) Cyclocreatine (mg/ml)
OVCAR*	0.6
H69	1.0
ME-180	1.8
DU-145	2.6
A2058	> 8.0

\*Only three doses: 0.5, 1.0 and 2.0 mg/ml

Except for the A2058 cells, which were unaffected by cyclocreatine, all cells tested exhibited a dose response to cyclocreatine. As the

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concentration of drug was increased, fewer cells achieved the minimum colony size in the time allowed.

The results of these experiments, together with the results obtained for continuous exposure to drug (see above), indicate that cyclocreatine inhibits the clonal growth of a variety of representative tumor cell lines. Furthermore, it is interesting to note that the cell lines most reproducibly inhibited by drug treatment as assayed by colony formation are those with the highest creatine kinase levels: H69 and ME-180 (Tables 1, 6 and 7). A2058, on the other hand, which expresses no detectable creatine kinase (data not shown), is unaffected by cyclocreatine. On the basis of the proven predictive value of soft agar colony assays, and the activity of cyclocreatine in this assay, it is concluded that cyclocreatine is an effective anti-tumor agent. These observations further suggest that tumors chemosensitive to cyclocreatine can be identified by screening biopsy samples for elevated creatine kinase activity, as compared with normal tissues.

#### Example 5

#### Inhibition of Colony Formation of Fresh Human Biopsy Tumor Samples in Soft Agar by Inhibitors of Creatine Kinase

##### Cells

Malignant ascites containing tumor cells were collected as part of a diagnostic workup or

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treatment of disease. Effusions were collected in preservative-free heparinized vacuum bottles, centrifuged at 150 X g for 10 minutes, and washed twice in Hank's balanced salt solution with 10 percent heat-inactivated fetal calf serum and 1% penicillin and streptomycin solutions. Viability of the cells was determined by trypan blue exclusion.

#### Drug Testing

Cyclocreatine and homocylcocreatine were added to 15 ml conical tubes. (The amount of drug added was determined by the volume of the drug stock added.) 2.0 ml of double-enriched Connaught Medical Research Laboratories (CMRL) medium was then added per tube. Finally, 0.5 ml of cells were added to each tube. Controls containing the corresponding solvent for each drug (i.e., water) were also tested. Tubes were vortexed and the contents were plated. 0.3 ml agar (measured in a 5 ml pipette) was drawn into the prepared 15 ml conical tube and mixed twice. The mixture was then drawn up and 1 ml was added to each plate. Plates were incubated at 37°C in 5-7% CO<sub>2</sub> and 100% humidity for a period of 14 days.

#### Media

##### ENRICHED CMRL 1066

For 500 mls of medium, to 400 ml CMRL 1066 (GIBCO #321-1535AJ) was added 75 ml Horse Serum (Hyclone), 10 ml Fetal Calf Serum (Hyclone), 10 ml

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Insulin (100 Units/ml), 5 ml Vitamin C (30 mM), 5 ml penicillin/streptomycin (5,000 U/ml, Baxter Cat. #T3512-30).

#### DOUBLE ENRICHED CMRL 1066

To 100 ml Enriched CMRL was added 1.5 ml asparagine (6.6 mg/ml) and 2.0 ml glutamine (200 mM). The pH was adjusted with 1 N HCl to give a final pH of 7.0 - 7.2 (~0.3 ml 1N HCl per 100 mls).

#### Plate Preparation

The underlayer was composed of 1 ml of 0.42% agar in plating medium. 2.5% Difco agar was diluted to 0.42% agar with the plating medium (40 ml Enriched McCoy's, 10 ml tryptic soy broth (3% in distilled H<sub>2</sub>O), 0.6 ml asparagine (6.6 mg/ml), 0.3 ml DEAE dextran (50 mg/ml), 0.4 ml glutamine (200 mM)). Ingredients were combined immediately before use: 10 ml of plating medium plus 2 ml 2.5% agar = 1/6 concentration (0.42%). This was mixed twice and 1 ml aliquots were added to twelve 35 mm petri dishes.

The top layer consisted of 1 ml of 0.25 agar. 2.5% agar was diluted to 0.25% agar with double Enriched CMRL 1066. 0.3 ml of 2.5% agar at 45 -50°C was added to each tube containing media, cells, and either drug or control solvent. The 0.3 ml agar was added with a 5 ml pipette. The resulting

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approximaty 3 ml's was mixed twice and then drawn up and 1 ml was added to each plate.

Effect of Cyclocreatine and Homocyclocreatine on Colony Formation of Freshly Collected Ovarian Ascites Cells

Ovarian Ascites tumor specimens were collected from a patient with ovarian cancer, were transferred to tubes in the presence of the appropriate drug dilution or control medium and were cultured in triplicate as described above. The number of colonies formed in the presence of 0.2, 2.0 and 20.0 mM homocyclocreatine (Table 8) and in the presence of 0, 0.067, 0.670 and 6.700 mM cyclocreatine (Table 9) are tabulated below.

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TABLE 8  
HOMOCYCLOCREATINE INHIBITS THE GROWTH OF FRESH HUMAN TUMOR CELLS  
(OVARIAN ASCITES CELLS) IN CLONOGENICITY ASSAYS

COMPOUND	DRUG CONC.	DISH 1	DISH 2	DISH 3 (# of colonies)	AVG	CONTROL	% RED
Homocyclo- creatine	0.2 mM	14	20	15	16	22	26%
"	2.0 mM	17	15	12	15	22	33%
"	20.0 mM	8	9	7	8	22	64%

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TABLE 9  
CYCLOCREATINE INHIBITS THE GROWTH OF FRESH HUMAN TUMOR CELLS  
(OVARIAN ASCITES CELLS) IN CLONOGENICITY ASSAYS

COMPOUND	DRUG CONC.	DISH 1	DISH 2	DISH 3	AVG	CONTROL	% RED
			(# of colonies)				
Cyclocreatine	0.067 mM	19	20	22	20	22	88
"	0.670 mM	19	13	18	17	22	248
"	6.700 mM	16	13	11	13	22	398

Treatment with 20 mM homocyclocreatine and 6.7 mM cyclocreatine reduced the number of colonies formed significantly. The two compounds showed less consistent growth inhibition of two additional biopsies. Nevertheless, these experiments with freshly derived tumor specimens confirm the antiproliferative effects of cyclocreatine observed above against established cell lines, indicating that the anti-proliferative activity of these compounds is not due to an anomalous property of the established cell lines. In addition, it is noted that the creatine kinase activity of these biopsies is unknown; therefore, the samples may not represent the most drug sensitive tumor types.

#### Example 6

#### A Creatine Kinase Inhibitor, Cyclocreatine, Reduces the Growth of Human Tumor Xenografts in Nude Mice

In previous studies investigating the ability of creatine kinase inhibitors to sustain ATP levels or delay rigor during ischemic episodes in muscle, cyclocreatine and other creatine analogs have been fed to mice, rats and chicks. All analogs were well-tolerated. Griffiths and Walker reported that chicks fed a diet containing 1 % cyclocreatine rapidly accumulated cyclocreatine-phosphate in breast muscle, a tissue rich in creatine kinase activity. Cyclocreatine also accumulated in heart and brain, although to a lesser extent. Chicks

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tolerated 1% cyclocreatine in the diet if an antibiotic (oxytetracycline) was also ingested, although these chicks grew more slowly than control chicks. Griffiths and Walker also reported that cyclocreatine was taken up by muscle, heart and brains in rats fed a diet containing 1% cyclocreatine (Griffiths, G.R. and J. B. Walker, J. Biol. Chem., 251: 2049-2054 (1976)).

Because cyclocreatine displayed reproducible antitumor activity in vitro (described above), was acceptably pure and was well-tolerated by animals, several experiments were performed to test whether cyclocreatine could inhibit the growth of human tumor xenografts in nude mice. Tumor cells selected on the basis of their in vitro sensitivity were implanted into mice by injection of cells grown in tissue culture or transplantation of tumor fragments grown in nude mice. After injection or implantation of cells or after formation of a palpable tumor, dietary (0.25 - 1.0%) drug was administered by mixture with chow. These amounts of cyclocreatine have been shown to accumulate in different organs in animals in mM concentrations; millimolar concentrations have been shown herein to have anti-proliferative activity in vitro. Tumor growth in the presence or absence of drug was followed by outside measurement of the growing tumor with ruler or caliper or by actual measurement of necropsied tumor.

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Cyclocreatine reduces the growth of newly  
established tumors derived from a human epidermoid  
carcinoma cell line in nude mice

Cells

ME-180 cells are derived from a metastasis to omentum of a human epidermoid carcinoma of the cervix (J. Nat. Cancer Inst. 45: 107-122, (1970)). Cells were purchased from the ATCC. The cells were passaged twice weekly for nine months before injection at a subcultivation ratio of 1:6, 1:8 by trypsinization. Medium consisted of 90% Minimum Essential Medium (Eagle's with Earle's Balanced Salts, without L-glutamine, J.R.H. Biosciences 51-412, EMEM), 10% fetal bovine serum, 100 U/ml, Penicillin/Streptomycin, 2 mM L-glutamine, 1 mM Na pyruvate (J.R.H. Biosciences). Cells were grown in 75 cm<sup>2</sup> flasks at 5% CO<sub>2</sub>, 37°C.

To harvest sufficient cells for the experiment, eight 225 cm<sup>2</sup> and ten 150 cm<sup>2</sup> confluent flasks of ME-180 cells were treated with 5 mls of 0.25% trypsin until the cells detached from the flask. Trypsin was inactivated with 10 mls of McCoy's medium + 10% FBS. Cells were collected by centrifugation for 5 mins at 1500 rpm. The supernatant was removed by aspiration and the pellet was resuspended in 40 ml of plain (no serum) media. The cell suspension was centrifuged again and the pellet was resuspended in 5 mls of serum free McCoy's media. A cell count was performed to determine cell number and viability.  $3.8 \times 10^7$

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cells were injected subcutaneously into one hind limb of each mouse. Injections were performed with a 1 cc syringe and a 26 G needle.

Tumors were measured from the exterior with a ruler held horizontally to the body of the mouse to determine the width, and perpendicularly to determine the height of the tumor. In some cases two tumors were visible. Tumor volume was estimated as the product of the width and the length squared divided by 2.

#### Mice

Nude (athymic) male mice from Charles River Breeding Labs were delivered as 8-10 week old mice. These mice were housed at the University of Massachusetts Animal Medicine Facility. Mice were acclimated for nineteen days before injection. To identify each mouse, a small hole was punched at a specific position of the outermost part of the right or left ear. The average weight of the mice at the beginning of the experiment was 28 grams.

#### Animal Husbandry Techniques

Mice were housed at the II Biotech Park Animal facility operated by the University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA. The animal room is kept at a temperature of 72°F, relative humidity of 40-70%. The animals are housed in polycarbonate mouse cages (Lab Products microisolator units, Lab Products, Inc., Maywood, NJ). Animals are housed on autoclaved bedding

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(Sani-Chip; P.J. Murphy, Inc., Montville, NJ). Animals are fed ad libitum Purina Diet 5010 autoclavable, (Purina, Inc.) and sterilized water. Animals are manipulated for husbandry under a laminar flow workstation (Nuair, Inc.). Animal cages are changed weekly.

#### Preparation of Drug

After injection of tumor cells, the mice were fed sterile ground meal from Farmers Exchange #5010 Picolab Mouse Chow. Meal was placed in jars holding 100 grams of chow, which were replaced twice weekly. Cyclocreatine (Ccr) was ground into the meal using the plunger section of a 60 cc syringe to reach a final concentration of 0.25%, 0.5%, 0.75%, or 1.0% cyclocreatine. This mix was added to sterile jars and placed in the cages and the standard pellet was removed.

#### Results

ME-180 cell-derived tumors were initiated in 25 nude mice by injection of  $3.8 \times 10^7$  cells per mouse subcutaneously in one hind limb of each mouse. Mice were returned to their original cages (5 mice per cage). 5 days after injection, tumors were palpable in each of the injected animals. On day 5, the test drug cyclocreatine was added as a supplement to the diet at 0.0 %, 0.25 %, 0.5 %, 0.75 %, and 1.0 % concentrations, with one selected concentration per cage. Mice were allowed to eat the mixture of drug

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and meal ad libitum. Tumor sizes and animal weights were recorded for the next two weeks.

Mice fed 0% cyclocreatine for two weeks gained on average  $2 \text{ g} \pm 1.4 \text{ g}$ , while mice fed 1 % cyclocreatine in the diet did not gain weight on average. No concentration tested induced a weight loss, on average. In several other experiments, different strains of nude mice and normal mice were observed to gain weight when fed 1% dietary cyclocreatine.

Addition of cyclocreatine to the diet of mice with preformed, palpable tumors inhibited tumor growth in a dose related manner. Tumors in mice fed 0% cyclocreatine grew on average  $19 \text{ mm}^3$  from week one to week three. Tumors in mice fed 0.25% cyclocreatine grew on average  $20 \text{ mm}^3$ . Tumors in mice fed 0.5% cyclocreatine grew only  $11 \text{ mm}^3$ . Tumors in mice fed 0.75% and 1.0% cyclocreatine did not grow and may have regressed (mean change in volume of  $-1$  and  $-6 \text{ mm}^3$ , respectively). It is concluded that cyclocreatine behaved as an effective antitumor drug in this experiment with no obvious toxicity (apart from a slight reduction of weight gain).

Cyclocreatine reduces the growth of ME-180 derived tumors in nude mice at the two week time point

Tumors were initiated by transplantation of tumor fragments derived from ME-180 cells injected into nude mice.  $1 \times 10^6$  ME-180 cells were injected subcutaneously at the dorsal midline near the neck

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of 2 nude CD-1 mice. The tumors were excised after 4 weeks of growth and were cut into approximately 1-2 mm<sup>3</sup> fragments with a scalpel blade, under sterile conditions. Mice were anesthetized and a 3 mm incision was made at the dorsal midline of each animal. A tumor fragment judged viable by the absence of color was placed into the incision with sterile forceps. The wound was closed with a wound clip, which was removed two weeks later. 30 CD-1 nude mice were inoculated with fragments of an ME-180-cell-derived tumor.

#### Mice

Nude (athymic) CD-1 male mice from Charles River Breeding Labs were delivered at 5 weeks of age.

#### Results

ME-180-derived tumors were initiated in 30 nude mice by transplantation of tumor fragments. After transplantation, mice were grouped 5 to a cage. The animals were started on a continuous drug regimen the same day. Two cages of control mice received 0% cyclocreatine, two cages of mice received 0.5% cyclocreatine chow, and two cages of mice received 1.0% cyclocreatine chow. After two weeks, five mice from each group were sacrificed. The remaining mice were sacrificed at week three. The mean volume of excised tumors for the control group was 19.7 mm<sup>3</sup> ( $\pm$  19.2 mm<sup>3</sup>). For animals receiving 0.5% drug, the average volume of the tumors reached 8.88 mm<sup>3</sup> ( $\pm$

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6.3 mm<sup>3</sup>), and for those receiving 1.0%, the average tumor volume was only 4.07 mm<sup>3</sup> ( $\pm$  1.5 mm<sup>3</sup>). One tumor in the control group did not form, introducing a large standard error in the measurement for the control group. Although the large variation in average tumor volume in the control group minimizes the statistical significance of this experiment, there is a clear dose response at this time point. The mice sacrificed at the three week time point showed no clear drug effect, with tumor volumes averaging 14.9 mm<sup>3</sup> ( $\pm$  19.4 mm<sup>3</sup>), 24.8 mm<sup>3</sup> ( $\pm$  19.6 mm<sup>3</sup>), and 11.5 mm<sup>3</sup> ( $\pm$  9.1 mm<sup>3</sup>) from 0.0%, 0.5%, and 1.0% cyclocreatine-fed animals, respectively.

Control animals grew modestly over the two to three week period of the experiment. The controls sacrificed at the two week time point had an average weight gain of 1.9 g (increasing from an average of 28.8 g to 30.7 g) at the time of sacrifice, while the group sacrificed after three weeks had grown on average 0.8 g (increasing from an average of 29.4 g to 30.2 g) at the time of sacrifice. Animals fed 0.5% cyclocreatine behaved similarly. Animals fed 1% cyclocreatine in their diet showed no significant weight gain or loss in either group.

The antitumor effect of 1% cyclocreatine on the growth of tumors at the two week time point is consistent with the antitumor activity observed in the previous experiment. The lack of effect observed at the three week time point may be due to the stepwise growth of tumors in this model. While the tumors in the control group may have reached a

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plateau after two weeks, the tumors in animals treated with 1% cyclocreatine may have caught up.

Cyclocreatine reduces the growth of human colon adenocarcinoma cells in nude mice

Cells

SW48 cells are human colon adenocarcinoma cells (Leibovitz et al., Cancer Res. 36: 4562-4569 (1976)). SW48 cells were purchased from the ATCC and are described further in Materials and Methods above. The cells were passaged twice weekly for nine months before injection at a subcultivation ratio of 1:3, 1:5. Feeding of cells was carried out as follows: media was removed by aspiration, 3 mls of fresh 0.25% Trypsin were added, plates were incubated for 1 minute until the cells were detached, and cells were added to 20 mls of fresh medium. Medium consisted of L15 Leibovitz, 90%; Fetal bovine Serum, 10%; Penicillin/Streptomycin, 1%; glutamine, 1%; Na pyruvate, 1%. Cells were incubated at 5% CO<sub>2</sub>, 37 °C.

Mice

Nude (athymic) CD-1 male mice from Charles River Breeding Labs were delivered at 5 wks of age. Mice were acclimated for 3 weeks before fragment insertion. Mice were identified during the course of the experiment for multiple measures of tumor size and mouse weight by the placement of a small hole punch in one ear. The average weight of the

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mice at the beginning of the experiment was 29 grams.

#### Tumor Measurement and Calculation

All tumors were initiated by transplantation of tumor fragment derived from SW48 cells injected into nude mice.  $1 \times 10^6$  SW48 cells were injected subcutaneously at the dorsal midline near the neck of 2 nude CD-1 mice. The tumors were excised after 4 weeks of growth and were cut into  $1-2 \text{ mm}^3$  fragments with a scalpel blade, under sterile conditions. Mice were anesthetized and a 3mm incision was made at the dorsal midline. A tumor fragment judged viable by the absence of color was placed into the incision with sterile forceps. The wound was closed with a wound clip that was removed two weeks later. 45 CD-1 nude mice were inoculated with fragments of an SW48-cell-derived tumor.

Two days (day 3) after insertion of the tumor fragment (day 1), cyclocreatine was introduced into the diet of 6 of the 9 cages of mice. 3 cages received 0.5% cyclocreatine, and 3 received 1.0% cyclocreatine mixed with their meal. On day 14, a 2 week time point was obtained following sacrifice and necropsy of 5 mice (one cage) per treatment group and control. The measurements of actual tumor volumes after necropsy are tabulated in Table 10. There was no dose-related response or significant statistical difference among the three groups.

Table 10Average Tumor Volumes (2 week time point, mm<sup>3</sup>)

<u>% cyclocreatine:</u>	<u>0%</u>	<u>0.5%</u>	<u>1.0%</u>
end (2 wk time point)	6.6	10.9	5.3
	(1.7)*	(6.2)	(2.5)

\* Values in parentheses are standard deviations of the measured values.

Beginning on day 19, outside tumor measurements were tracked for the remaining 6 cages of mice, 2 cages per treatment and control group. On day 45, the experiment was terminated and the actual tumor volumes were calculated upon necropsy. The average tumor volumes determined for each of the days are listed below in Table 11 and values for days 19-30 post-drug addition are illustrated graphically in Figure 12. In Figure 12, the mean tumor volume (mm<sup>3</sup>) is plotted against the time (days post-drug addition) for each treatment group.

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Table 11Average Tumor Volumes (time course, mm<sup>3</sup>)

<u>% cyclocreatine:</u>	<u>0.0%</u>	<u>0.5%</u>	<u>1.0%</u>
day 19	92.1 (84)*	132.6 (87.7)	33.5 (18.6)
day 23	123.5 (92.7)	125.4 (77.6)	38.1 (28.6)
day 26	168.8 (119)	196.0 (143)	49.7 (35.1)
day 30	278.8 (267.1)	332.6 (252.9)	66.3 (59.7)
day 34	424.6 (335.2)	398.9 (301)	101.8 (89.8)
day 37	521.8 (459)	487.8 (426.1)	131.5 (128.4)
day 41	478.4 (447)	710.5 (701)	169.5 (143.3)
end (day 43) (actual)	650.6 (579.8)	851.9 (950)	221.1 (176.4)

\* Values in parentheses are standard deviations.

It is concluded that there is a significant reduction in growth of SW48-derived tumors in nude mice fed 1% dietary cyclocreatine ad libitum. The reduction in tumor growth is apparent in each exterior measurement from day 19 to 41 and in the actual measurement at autopsy. In contrast to the tumors initiated in the animals fed with 1% drug, the tumors in the animals fed with 0.5% drug exhibited no obvious changes in growth pattern when compared to the 0% cyclocreatine controls.

In contrast to the antitumor effect of the 1% cyclocreatine diet on tumor growth, there is no

obvious toxicity of the drug. There was no significant reduction in animal weight gain in the group treated with 1% cyclocreatine as compared with the control (0%), although there was a modest difference between the means.

Table 12

Average Mouse Weight (g)

<u>% cyclocreatine:</u>	<u>0.0%</u>	<u>0.5%</u>	<u>1.0%</u>
start (day 6)	28.6 (1.3)*	30.2 (1.8)	29.2 (2.4)
end (day 41)	29.9 (3.9)	31.8 (3.9)	28.4 (4.5)

\* Values recorded in parentheses are standard deviations.

Cyclocreatine inhibits the growth of established (> 50 mm<sup>3</sup>) ME-180 cell derived tumors in nude mice

Cells

ME-180 cells are human cervical epidermoid carcinoma, derived from a metastasis to omentum, and are described further in Materials and Methods above (J. Nat. Cancer Inst., 45: 107-122, 1970).

Mice

Nude (athymic) male mice from Charles River Breeding Labs were delivered at 4-5 weeks of age. Mice were acclimated for nine days before injection.

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### Results

38 mice in eight cages (5 mice in each of 7 cages, and 3 mice in one cage) were each injected subcutaneously at the dorsal midline with  $2 \times 10^6$  cells in 0.2 ml, using a 1 cc syringe and a 26 G needle. Tumors were allowed to grow for 24 days after which cages of mice were selected to receive 0%, 0.5%, 0.75% or 1.0% cyclocreatine mixed in their diet as described above. 2 cages containing 5 mice each received no drug and served as controls.

Only 6 of the 10 mice in the control cages qualified for the experiment by having tumors greater than  $50 \text{ mm}^3$  ( $n = 6$ ; average tumor volume =  $146.3 \text{ mm}^3 \pm 77 \text{ mm}^3$ ). The other 4 mice were left in the cages in order to maintain the feeding patterns of the experimental mice.

Two cages were selected to receive 0.5% cyclocreatine. Only 3 of the eight mice had tumors greater than  $50 \text{ mm}^3$  on day 24 ( $n = 3$ ; average tumor volume =  $86.9 \text{ mm}^3 \pm 25.2 \text{ mm}^3$ ). Two cages containing 9 mice received 0.75% dietary cyclocreatine. Only 3 of these mice qualified for the experiment ( $n = 3$ ; average tumor volume =  $147.5 \text{ mm}^3 \pm 84 \text{ mm}^3$ ). Two cages totalling 10 mice were selected to receive 1% cyclocreatine. 7 of these 10 mice qualified for the experiment ( $n = 7$ ; average tumor volume =  $165.4 \text{ mm}^3 \pm 98 \text{ mm}^3$ ). Cages receiving 0% and 1% drug were chosen to maximize the number of qualifying mice and to match starting average tumor volumes as closely as possible for a meaningful comparison. Mice were fed drug for 42 days.

During the course of the experiment, several mice died, including one from the 0% group, one from the 0.75% cyclocreatine-fed group, and two from the 1%

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cyclocreatine-fed group. (The animal in 0.75% group appeared sickly and was sacrificed before the drug regimen was started.) The deaths appeared random and were without a known cause. The tumor volumes of the remaining mice were determined and are tabulated below in Table 13.

Table 13

<u>Average Volume of Tumor (mm<sup>3</sup>)</u>				
<u>% cyclocreatine:</u>	<u>0.0%</u>	<u>0.5%</u>	<u>0.75%</u>	<u>1.0%</u>
start	146 (77)*	86.9 (25.2)	147.5 (84)	165.4 (98)
end	1224.5 (1046)	873 (905)	763.4 (951)	657.6 (603)

\* Values in parentheses are standard deviations.

Table 14

<u>Average Change in Tumor Volume (mm<sup>3</sup>)</u>				
<u>% cyclocreatine:</u>	<u>0%</u>	<u>0.5%</u>	<u>0.75%</u>	<u>1.0%</u>
end	1078.2	786	615.9	492.2

As can be seen from this data, there is a dose-related reduction in tumor growth in cyclocreatine-fed animals. Tumors in animals fed 1% cyclocreatine grew 2.2 times less than tumors in control animals.

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The volumes of the tumors that were too small at the start of the experiment to qualify (less than  $50 \text{ mm}^3$ ) were also measured during the course of the experiment. In Tables 15 and 16, these volumes are included in determining the mean tumor volumes (Table 15) and mean change in tumor volume as of the end of the experiment (Table 16) for mice subjected to the various drug regimens. The average change in tumor volumes ( $\text{mm}^3$ ) are also displayed in the form of a graph in Figure 13, in which the average change in tumor volume for different drug regimens is plotted against time (days post-drug administration).

Table 15Average Volume of Tumor ( $\text{mm}^3$ )

% cyclocreatine:	0.0%	0.5%	0.75%	1.0%
start	106 (85.6) *	54.8 (30.4)	65.5 (75.4)	122.5 (100.2)
end	1033 (1049)	752.1 (605)	461.4 (620)	464.9 (531)

\* Values recorded in parentheses are standard deviations.

Table 16Average Change in Tumor Volume ( $\text{mm}^3$ )

% cyclocreatine:	0.0%	0.5%	0.75%	1.0%
end	927	697.3	395.5	342.4

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These results mirror those calculated using only the tumors grown to a size greater than 50 mm<sup>3</sup> at the start of the experiment. There appears to be a dose-related reduction of tumor volume in the cyclocreatine-fed animals. It is concluded that cyclocreatine reduces the growth of both "established" and "unestablished" tumors.

The weight of each mouse was monitored during the course of the experiment. The average weights determined are shown below in Table 17.

Table 17

<u>Average Weight of Mice</u>				
<u>% cyclocreatine:</u>	<u>0.0%</u>	<u>0.5%</u>	<u>0.75%</u>	<u>1.0%</u>
Start	28.9 (1.1)*	28.4 (2.3)	28.7 (1.0)	29.4 (1.0)
End	31.4 (1.3)	33.8 (3.7)	28.7 (2.1)	27.3 (3.3)

\* Values recorded in parentheses are standard deviations.

A slight decrease in weight that is not statistically significant was observed in the 1% drug-fed group. No other sign of potential drug-related toxicity was observed.

It is concluded from these examples that cyclocreatine has anti-tumor activity in vivo in human tumor xenograft models. The absence of gross toxicity is consistent with in vitro results with normal cell lines and with other feeding experiments

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done by Walker and colleagues in investigations of ischemia. Cyclocreatine apparently possesses both the potency and specificity necessary in an effective antiproliferative, anticancer drug.

CLAIMS

1. Use of a drug capable of inhibiting purine metabolic enzyme activity, for the manufacture of a medicament for inhibiting growth or metastasis of a mammalian cell in which activity of purine metabolic enzyme (e.g. creatine kinase, adenylate cyclase, adenylate kinase, adenosine deaminase or adenosine kinase) is elevated, by e.g. a DNA tumor virus or a DNA tumor virus factor.
2. Use according to Claim 1, wherein the drug is selected from the group consisting of:
  - a) cyclocreatine;
  - b) homocyclocreatine;
  - c) 1-carboxymethyl-2-iminohexahydro-pyrimidine;
  - d) guanidinoacetate; and
  - e) carbocreatine.
3. Use of a drug selected from the group consisting of:
  - a) cyclocreatine;
  - b) homocyclocreatine;
  - c) 1-carboxymethyl-2-iminohexahydro-pyrimidine;
  - d) guanidinoacetate; and
  - e) carbocreatine;

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for the manufacture of a medicament for inhibiting transformation, growth or metastasis of a mammalian cell, for example a cell in which creatine kinase activity is elevated by e.g. a DNA tumor virus or a DNA virus factor.

4. Use according to any one of Claims 1, 2 and 3 wherein the DNA tumor virus is selected from the group consisting of papovaviruses, Herpes viruses, and adenoviruses.
5. Use according to Claim 4 wherein the papovavirus is a human papillomavirus and the DNA tumor virus factor is a product encoded by the E7 gene of human papillomavirus.
6. Use according to Claim 5 wherein the human papillomavirus is selected from the group consisting of human papillomavirus 16, human papillomavirus 19, human papillomavirus 31 and human papillomavirus 33.
7. Use according to Claim 4 wherein the DNA tumor virus is an adenovirus and the DNA tumor virus factor is an adenovirus E1a gene product or an oncogenic factor induced by adenovirus.
8. Use of a drug selected from the group consisting of:
  - a) cyclocreatine;
  - b) homocyclocreatine;

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c) 1-carboxymethyl-2-iminohexahydro-pyrimidine;

d) guanidinoacetate; and

e) carbocreatine;

for the manufacture of a medicament for inhibiting transformation, growth or metastasis of a mammalian cell, for example a cell in which the activity of a purine metabolic enzyme is elevated.

9. A method of inhibiting growth or metastasis of a mammalian cell in which activity of a purine metabolic enzyme is elevated, comprising inhibiting activity of the purine metabolic enzyme by contacting the mammalian cell with a drug capable of inhibiting purine metabolic enzyme activity, under appropriate conditions.
10. The method of Claim 9 which is a method of inhibiting growth or metastasis of a mammalian cell in which activity of a purine metabolic enzyme is elevated by a DNA tumor virus, a DNA tumor virus factor or other factor which has an equivalent effect on the mammalian cell.
11. The method of Claim 9 wherein the purine metabolic enzyme is selected from the group consisting of: creatine kinase, adenylate cyclase, adenylate kinase, adenosine deaminase, and adenosine kinase.

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12. The method of Claim 11 wherein the purine metabolic enzyme is creatine kinase and the drug is selected from the group consisting of:
- a) cyclocreatine;
  - b) homocyclocreatine;
  - c) 1-carboxymethyl-2-iminohexahydropyrimidine;
  - d) guanidinoacetate; and
  - e) carbocreatine.
13. The method of Claim 9 wherein the appropriate conditions are conditions appropriate for the drug to pass into the mammalian cell, thereby resulting in passage of the drug into the mammalian cell.
14. The method of Claim 9 wherein the appropriate conditions are conditions appropriate for the drug to remain at or within the mammalian cell membrane, thereby resulting in the drug's remaining at or within the mammalian cell membrane.
15. The method of Claim 10 wherein inhibiting activity of a purine metabolic enzyme is carried out by inhibiting the purine metabolic enzyme whose activity in the mammalian cell is elevated.
16. The method of Claim 15 wherein the DNA tumor virus is selected from the group consisting of

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papovaviruses, Herpes viruses, and adenoviruses.

17. The method of Claim 16 wherein the papovavirus is a human papillomavirus and the DNA tumor virus factor is a product encoded by the E7 gene of human papillomavirus.
18. The method of Claim 17 wherein the human papillomavirus is selected from the group consisting of human papillomavirus 16, human papillomavirus 19, human papillomavirus 31 and human papillomavirus 33.
19. The method of Claim 16 wherein the DNA tumor virus is an adenovirus and the DNA tumor virus factor is an adenovirus E1a gene product or an oncogenic factor induced by adenovirus.
20. A method of inhibiting in a mammal the metastasis of a mammalian cell in which activity of a purine metabolic enzyme is elevated, comprising administering to the mammal a compound capable of inhibiting the activity of the purine metabolic enzyme.
21. A method of Claim 20 wherein a DNA tumor virus, DNA tumor virus factor or other factor has acted to increase the activity of the purine metabolic enzyme.

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22. The method of Claim 21 wherein the purine metabolic enzyme is creatine kinase.
23. A method of inhibiting in a mammal the growth of a transformed cell in which the activity of a purine metabolic enzyme is elevated, comprising administering to the mammal a compound which inhibits the activity of the purine metabolic enzyme.
24. The method of Claim 23 wherein a DNA tumor virus, DNA tumor virus factor or other factor has acted to increase the activity of the purine metabolic enzyme.
25. The method of Claim 24 wherein the purine metabolic enzyme is creatine kinase.
26. A method of inhibiting growth or metastasis of a mammalian cell in which activity of a purine metabolic enzyme is elevated, comprising contacting the mammalian cell with a drug selected from the group consisting of:
  - a) cyclocreatine;
  - b) homocyclocreatine;
  - c) 1-carboxymethyl-2-iminohexahydro-pyrimidine;
  - d) guanidinoacetate; and
  - e) carbocreatine.

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27. A method of determining the sensitivity of a tumor, present in an individual, to cyclocreatine comprising determining the level of creatine kinase activity in a specimen of the tumor or serum sample obtained from the individual, wherein elevated creatine kinase activity in the specimen of the tumor or serum sample or a high level of creatine kinase activity is indicative of sensitivity to cyclocreatine.

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FIG. 1

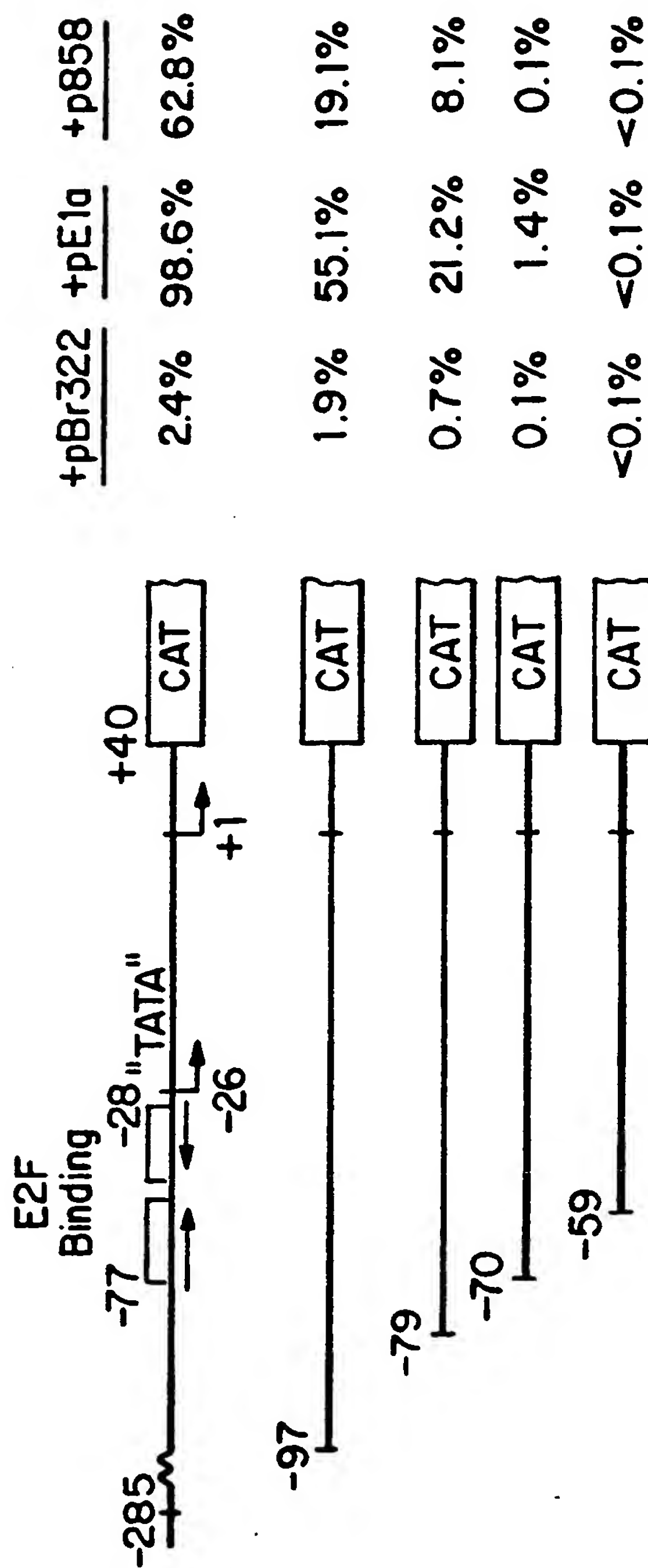


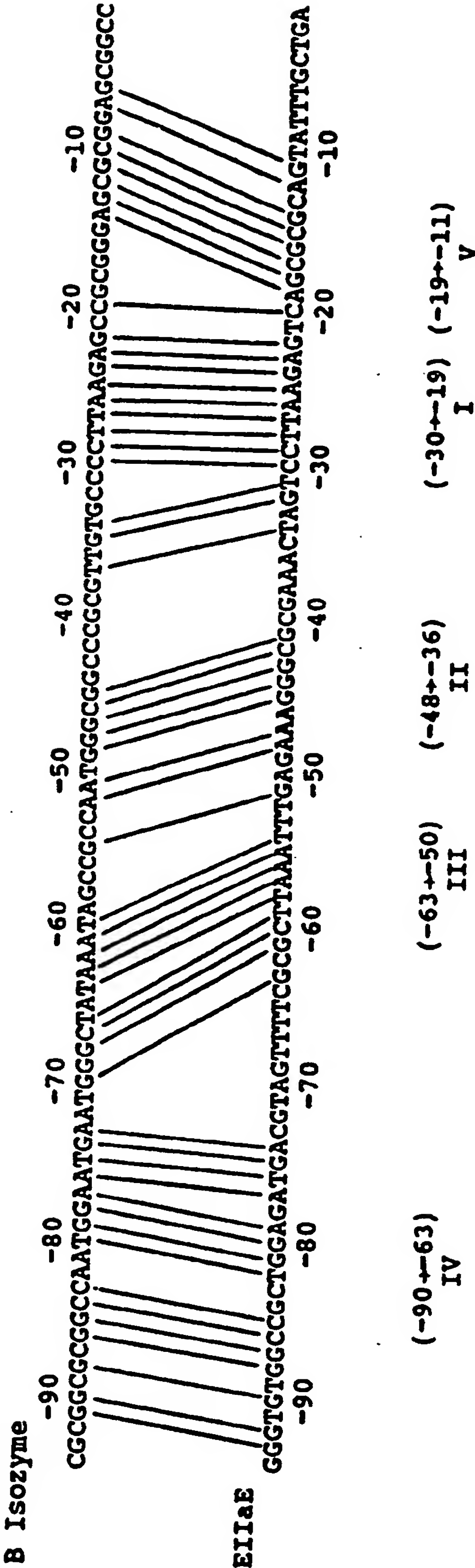
Diagram illustrating the amino acid sequence alignment of the E7 protein of Ad5 (Ad5 E1a, 289 AA) and HPV-16-E7 (97 AA). The diagram shows the alignment of the two proteins, with residues numbered 1 to 289 for Ad5 E1a and 1 to 97 for HPV-16-E7. The alignment is shown in a schematic manner, with boxes representing individual amino acids and dashed lines indicating the alignment. The HPV-16-E7 sequence is shown in a separate box, with residues numbered 1 to 97. The alignment shows that the HPV-16-E7 protein is highly similar to the E7 protein of Ad5, with many identical residues and conserved motifs.

Ad5 E1a (289 AA)

HPV-16-E7 (97 AA)

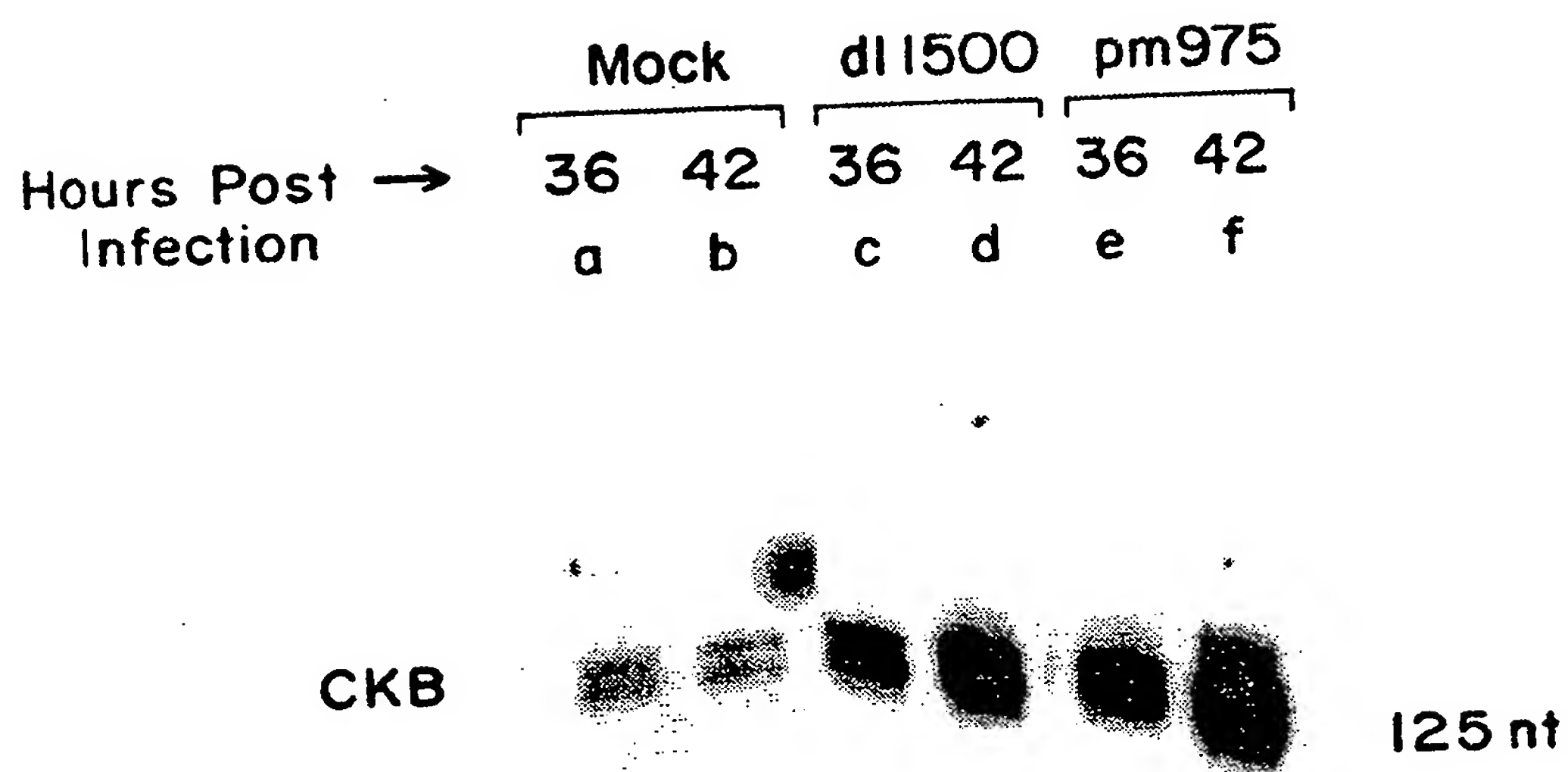
HPV-16 E7  
 HPV-31 E7  
 HPV-33 E7  
 HPV-18 E7  
 HPV-6 E7  
 HPV-11 E7

FIG. 3



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## FIG. 4



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FIG. 5A

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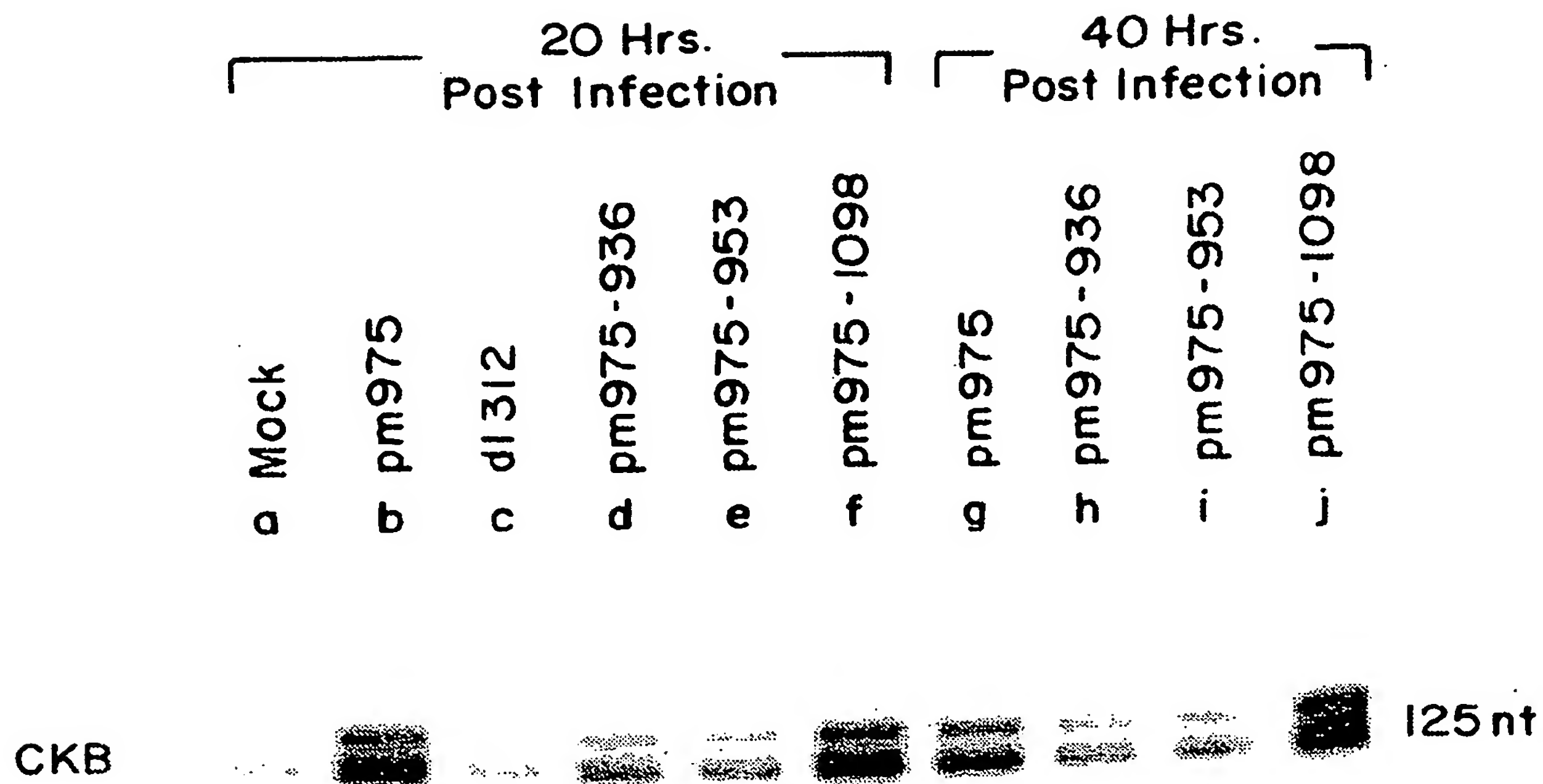
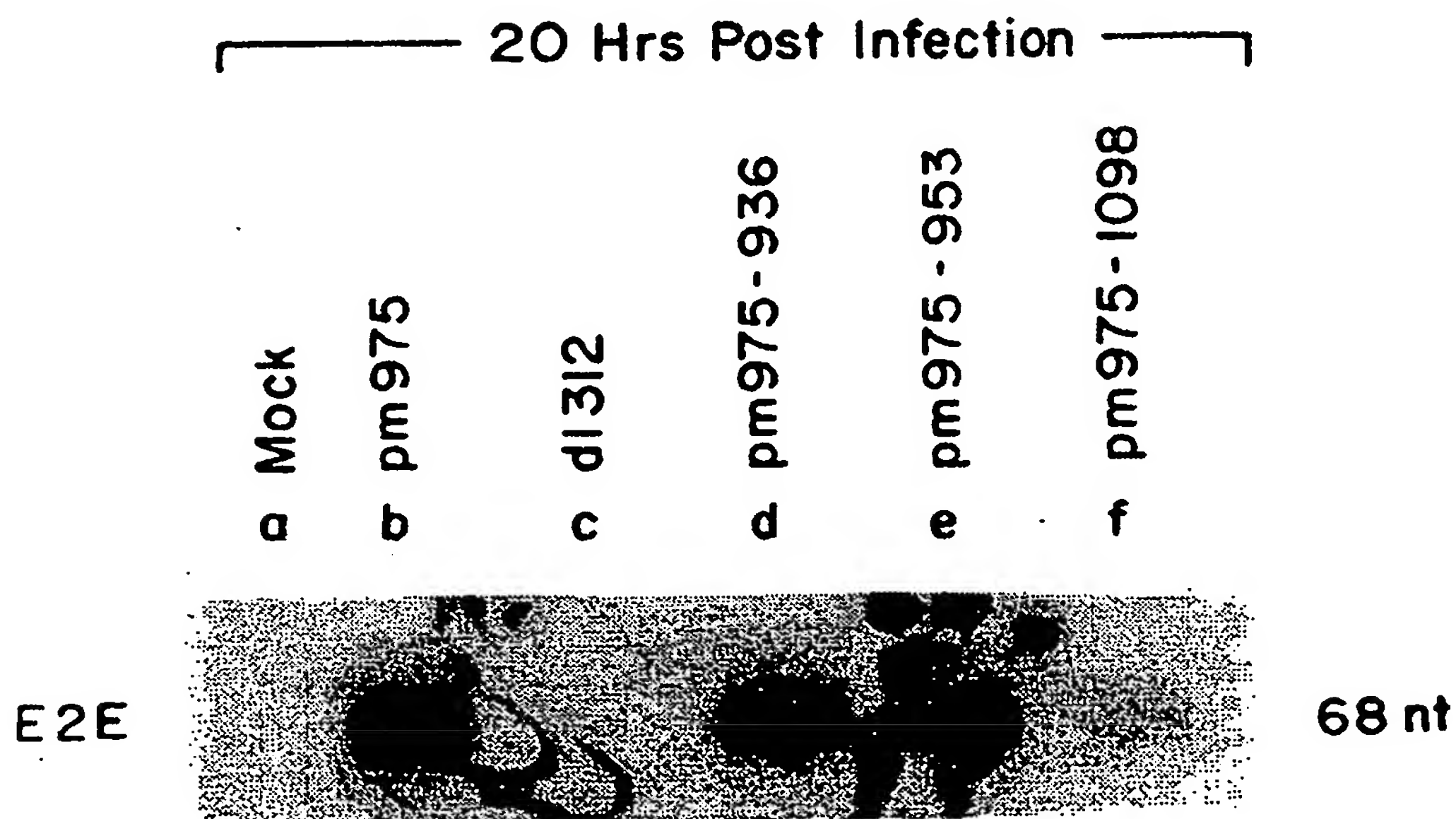


FIG. 5B



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FIG. 6A

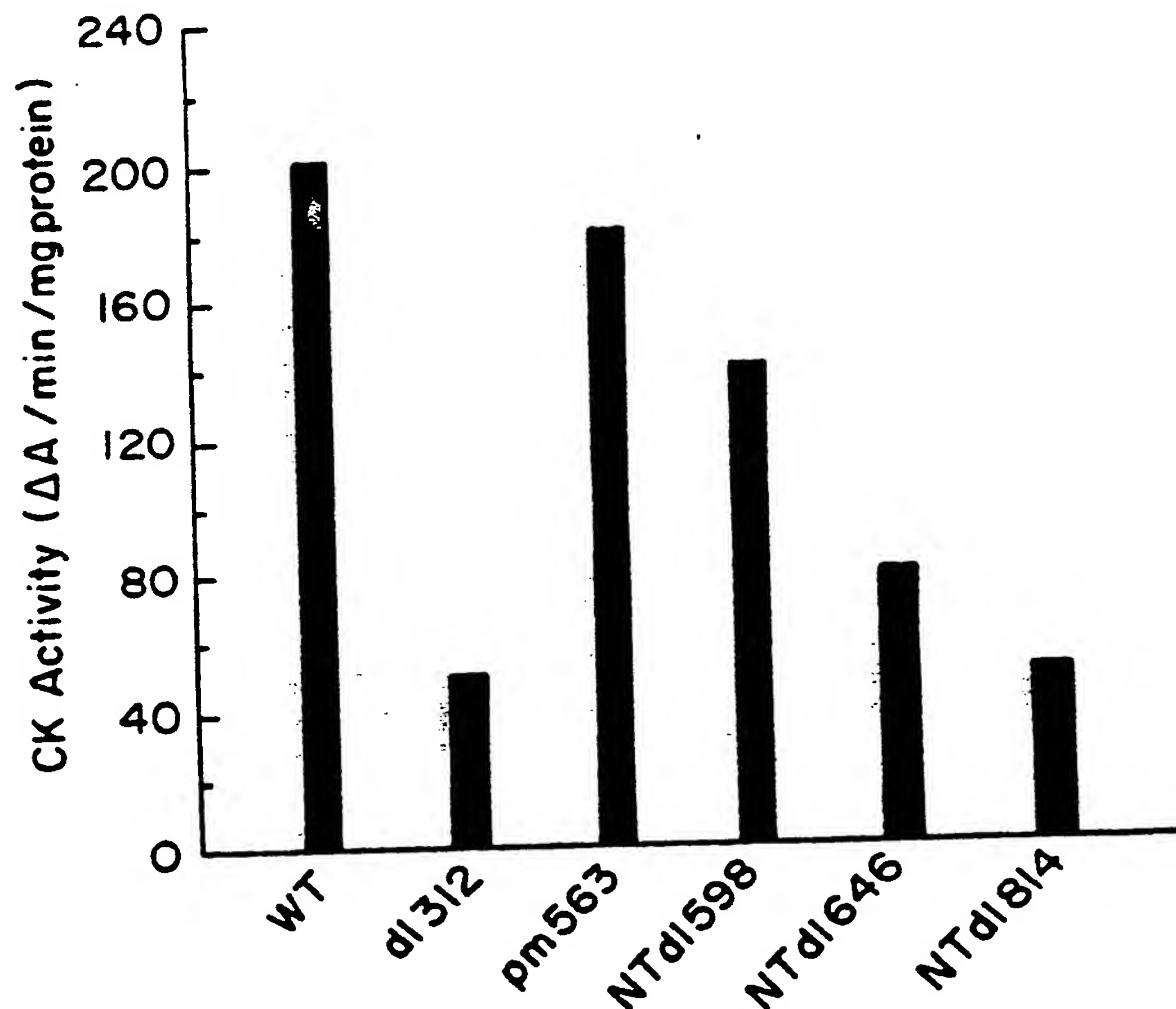


FIG. 6B

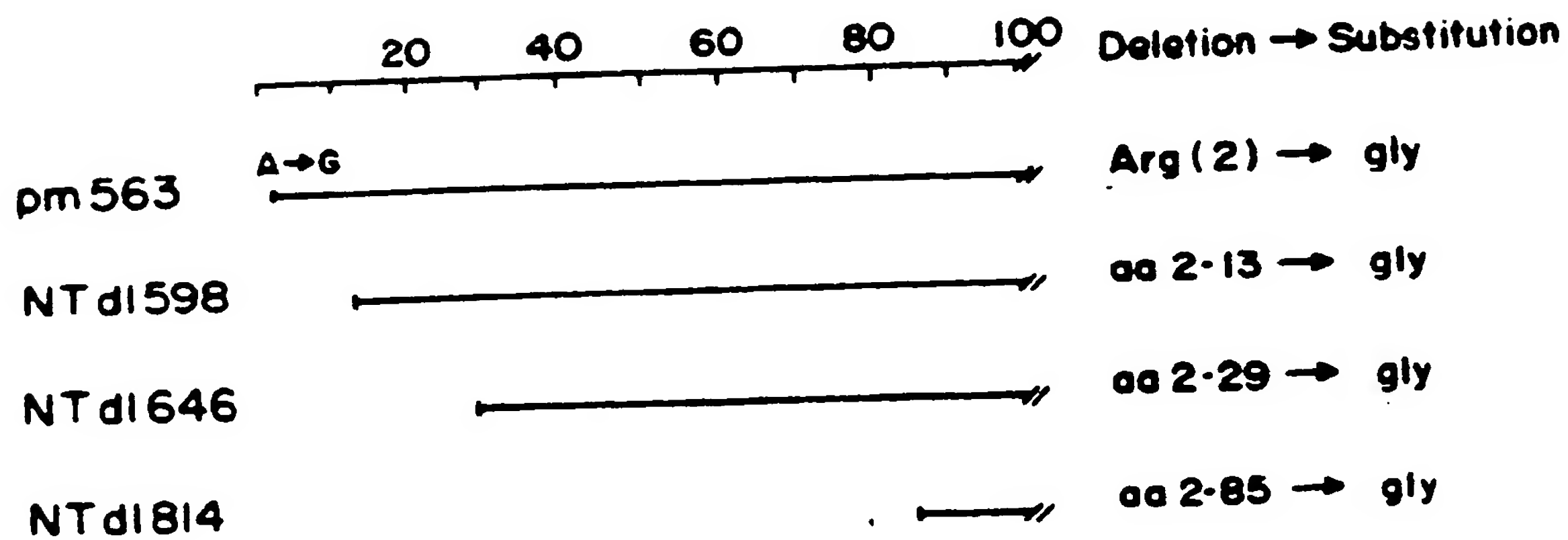


FIG. 7

Sequence of phck111f

1 CAGATCGAAA CGCTCTTCAA GTCTAAGAAC AACTACGAGT TCATGTGGAA CCTCACCTG  
61 GGCTACATCC TCACCTGCCC CTCCAACCTG GGCACGGGGC TCGGGGCAGG CGTGACATC  
121 AAGCTGCCCC ACCTGGGCCA GCACCAGAAG TTCTCCCAGG TGCTCAAGCG GCTGCGGCTT  
181 CAGAAAGCAG GCACAGGTGA GCAGGGCAGG TGCTGCGGCT TCCCGTGGCC TTTGGGCAGC  
241 CCTGTTTCCT CCGCCCTGAC TTGCTGTCCC CAGGCGGTGT GGACAAGGCT GCGGTGGCGG  
301 GGTCTTTCGA CGTCTCCAAC GCTGACCGCC TGGGCTTCTC AGAGGTGGAG CTGGTGCAGA  
361 TGCTGGACGG AGTGAAGCTG CTCATCGAGA TGGAACAGCG GCTGGAGCAG GGCCAGGCCA  
421 TCGACGACCT CATGCCCTGCC CAGAAATGAA GCCCGGCCCA CACCGACACA GCCCTGCTGC  
481 TTCCTAACTT ATTGCCCTGG CAGTGcACCA TGCACCCCTGA TGTTCGCCGT CTGACGCCCT  
541 TAGCCTTGCT GTAGAGACTT CCGTCACCTT GGTAGAGTTT ATTTtGAT

Sequence displayed from position 1 to end (position 589)

Sequence numbered from position 1



## DUI45 INHIBITION BY HOMOCYCLOCREATINE

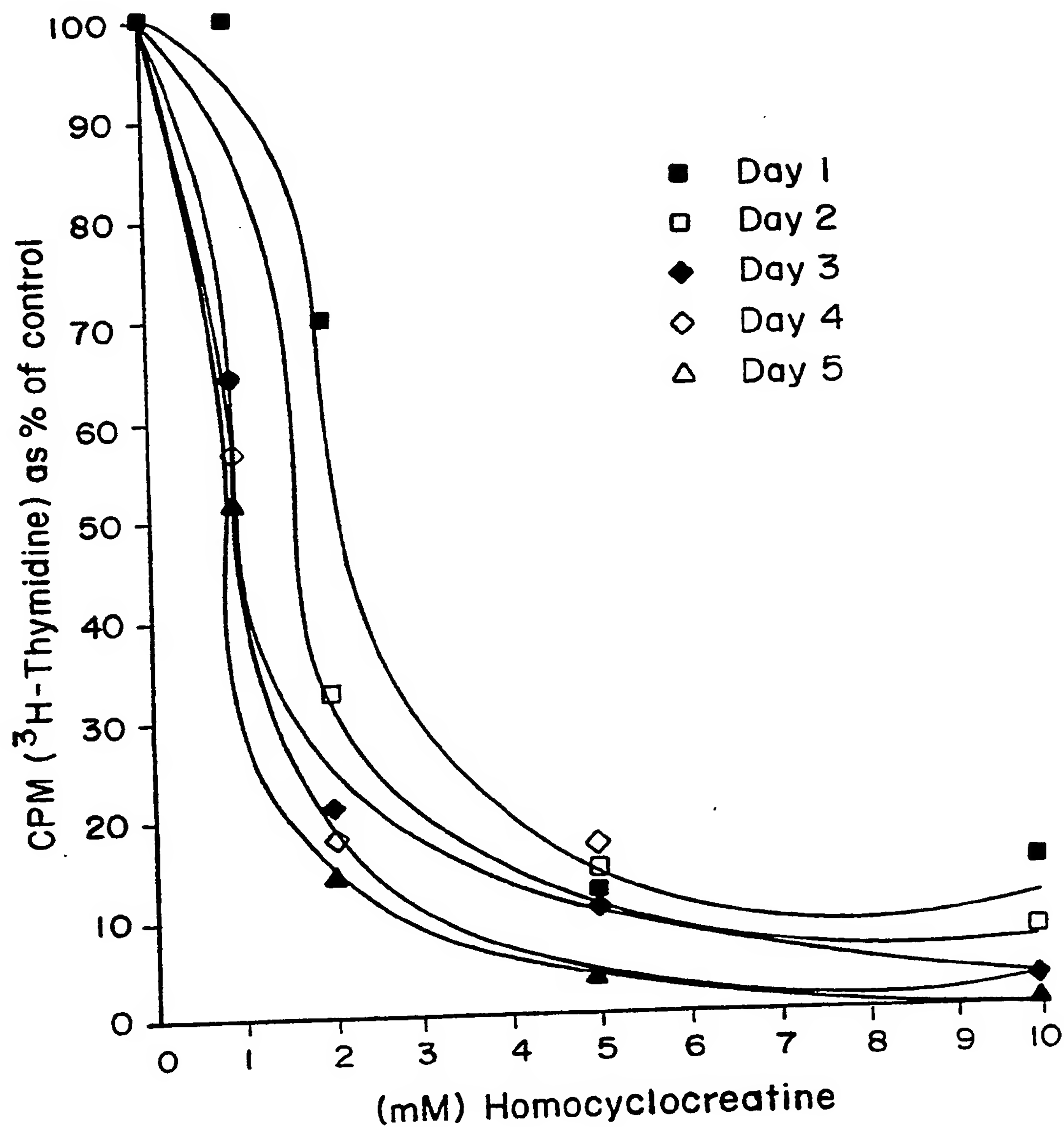


FIG. 8

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# Cyclocreatine Inhibits the Growth of Multiple Colon Carcinoma Cell Lines (7 Day Exposure)

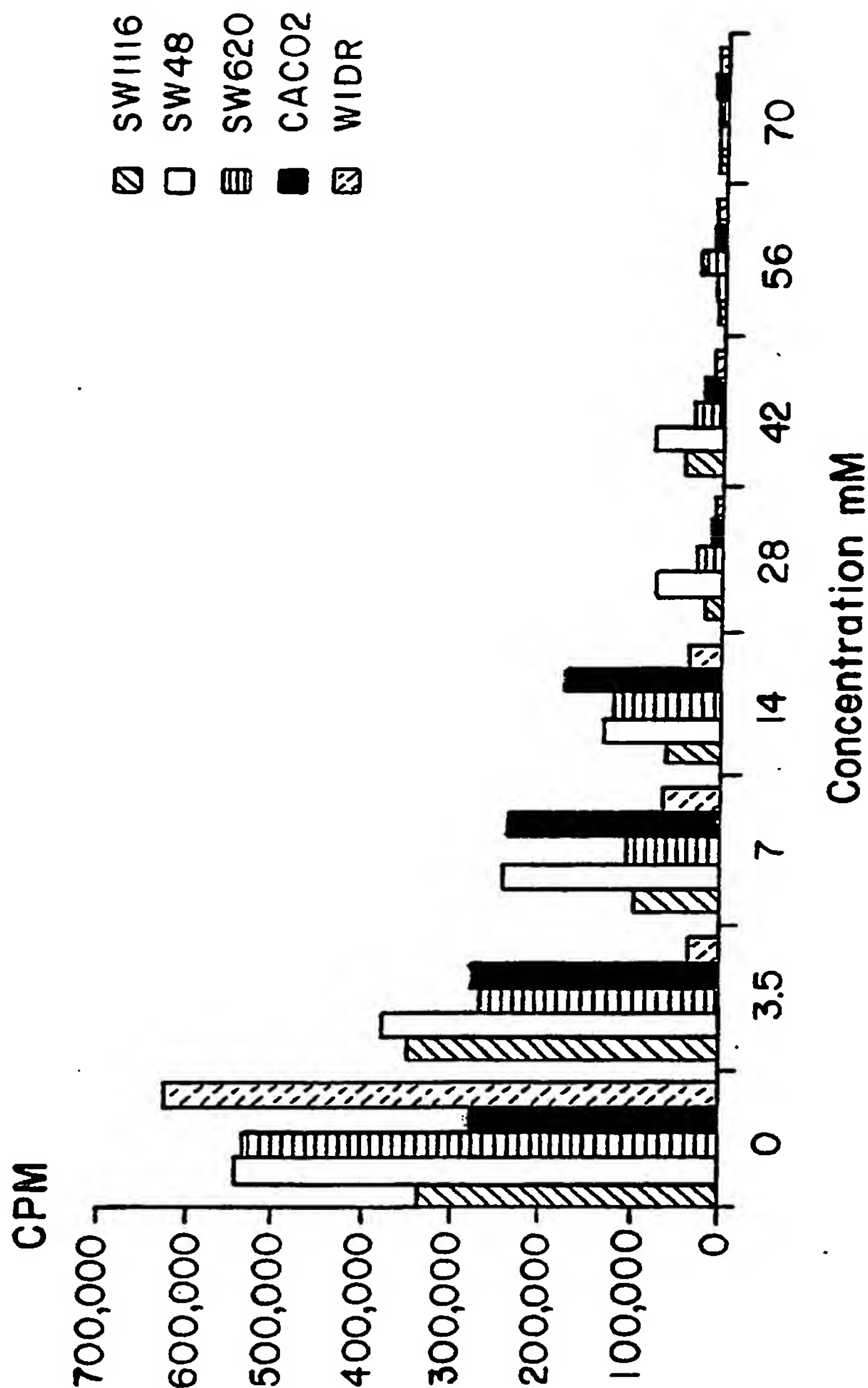


FIG. 9

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# Effects of 4mg/ml Homocyclocreatine on Various Cell Lines

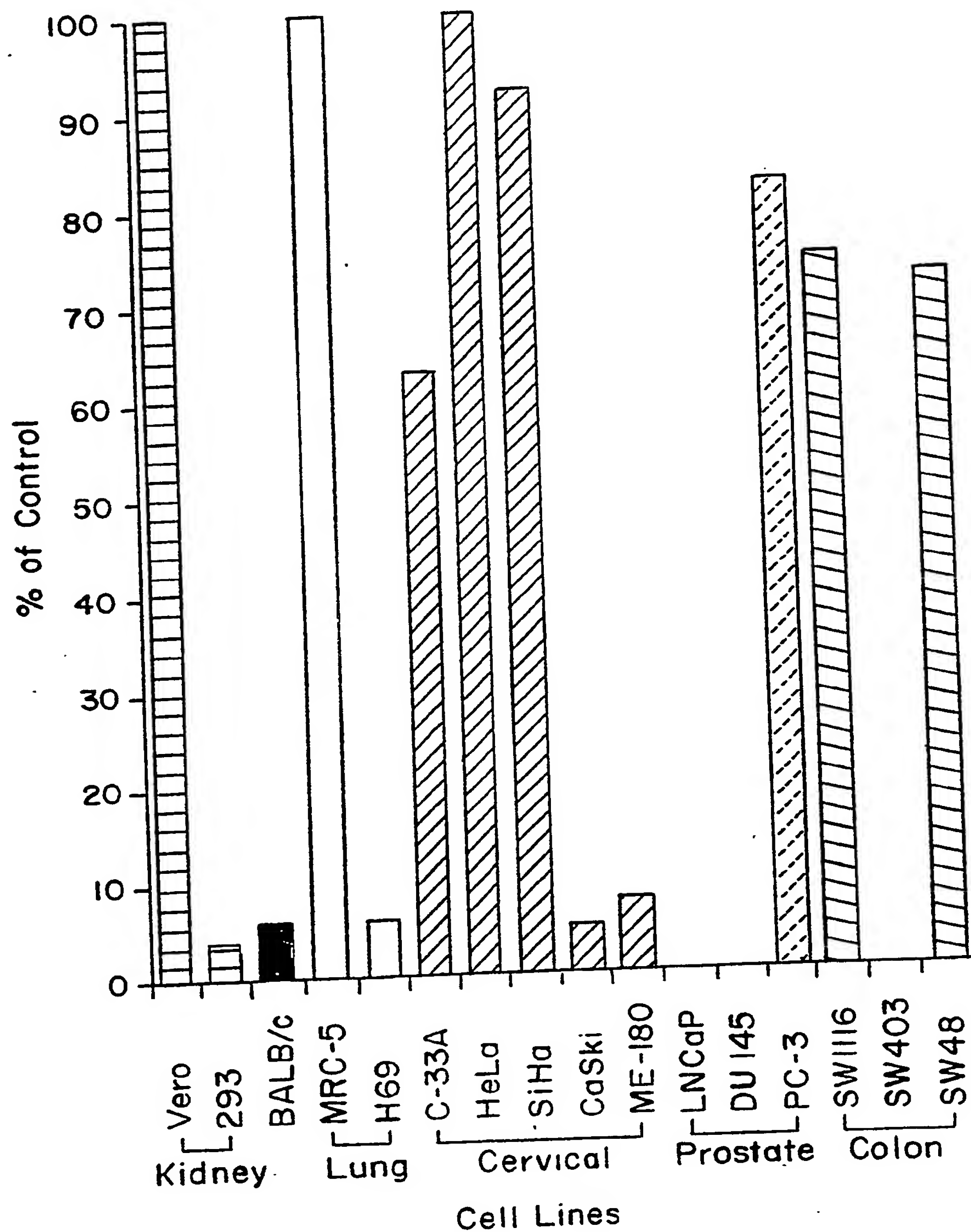


FIG. 10A  
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Effects of  
4mg/ml 1-carboxymethyl-2-imino-hexahydropyrimidine  
on Various Cell Lines

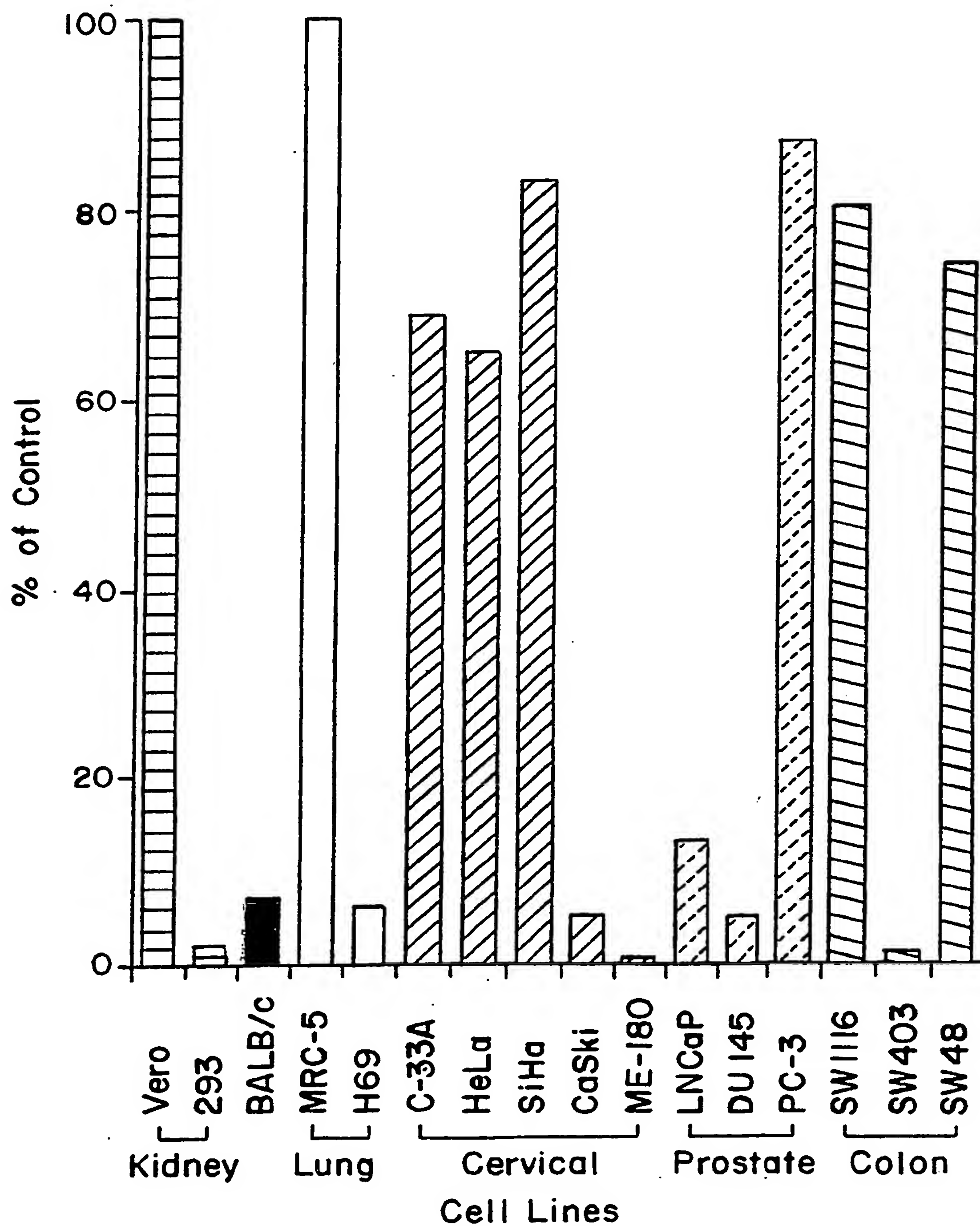


FIG. 10B  
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# Effects of 4mg/ml Cyclocreatine on Various Cell Lines

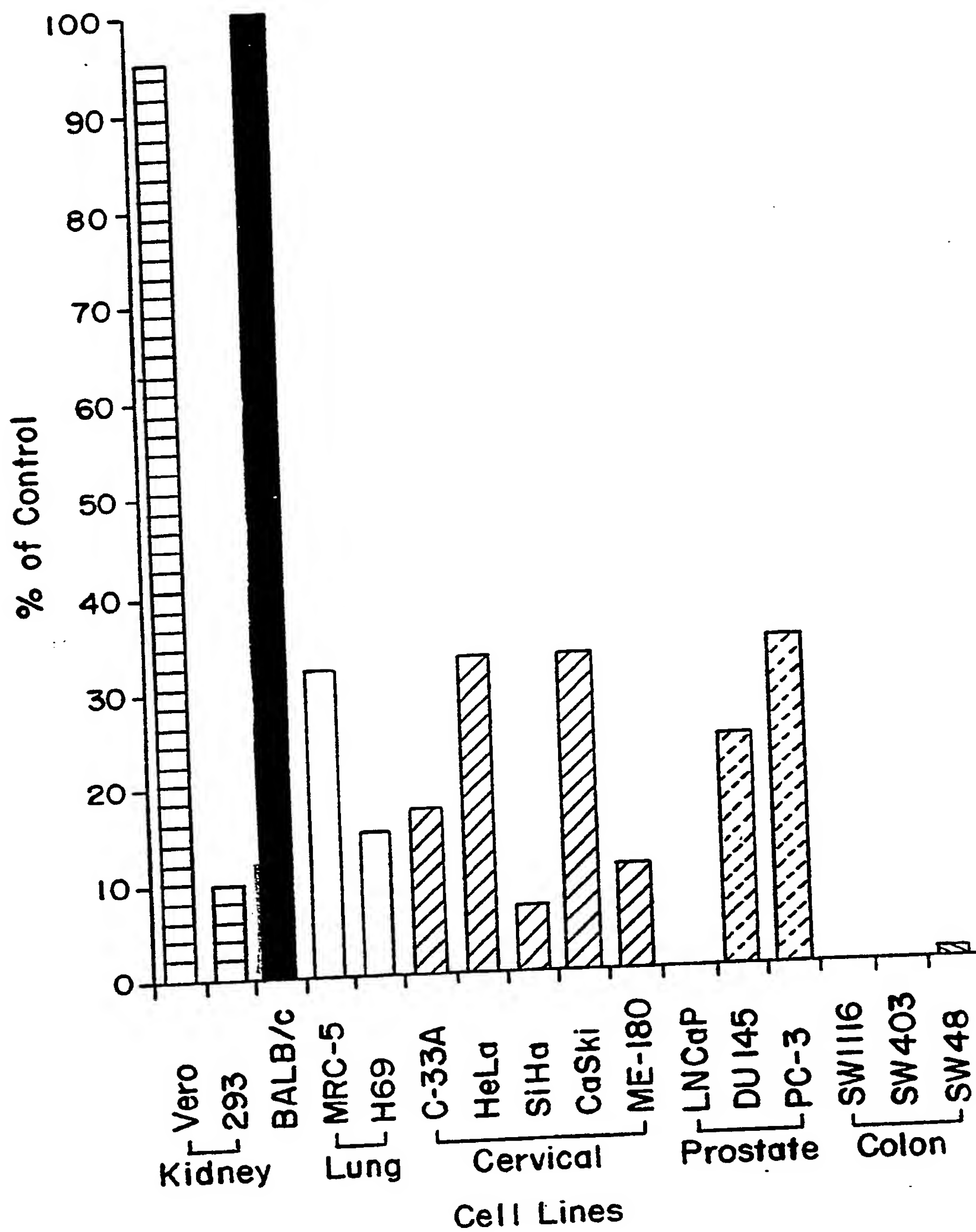


FIG. 10C

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## Effects of 4mg/ml Guanidino Acetic Acid on Various Cell Lines

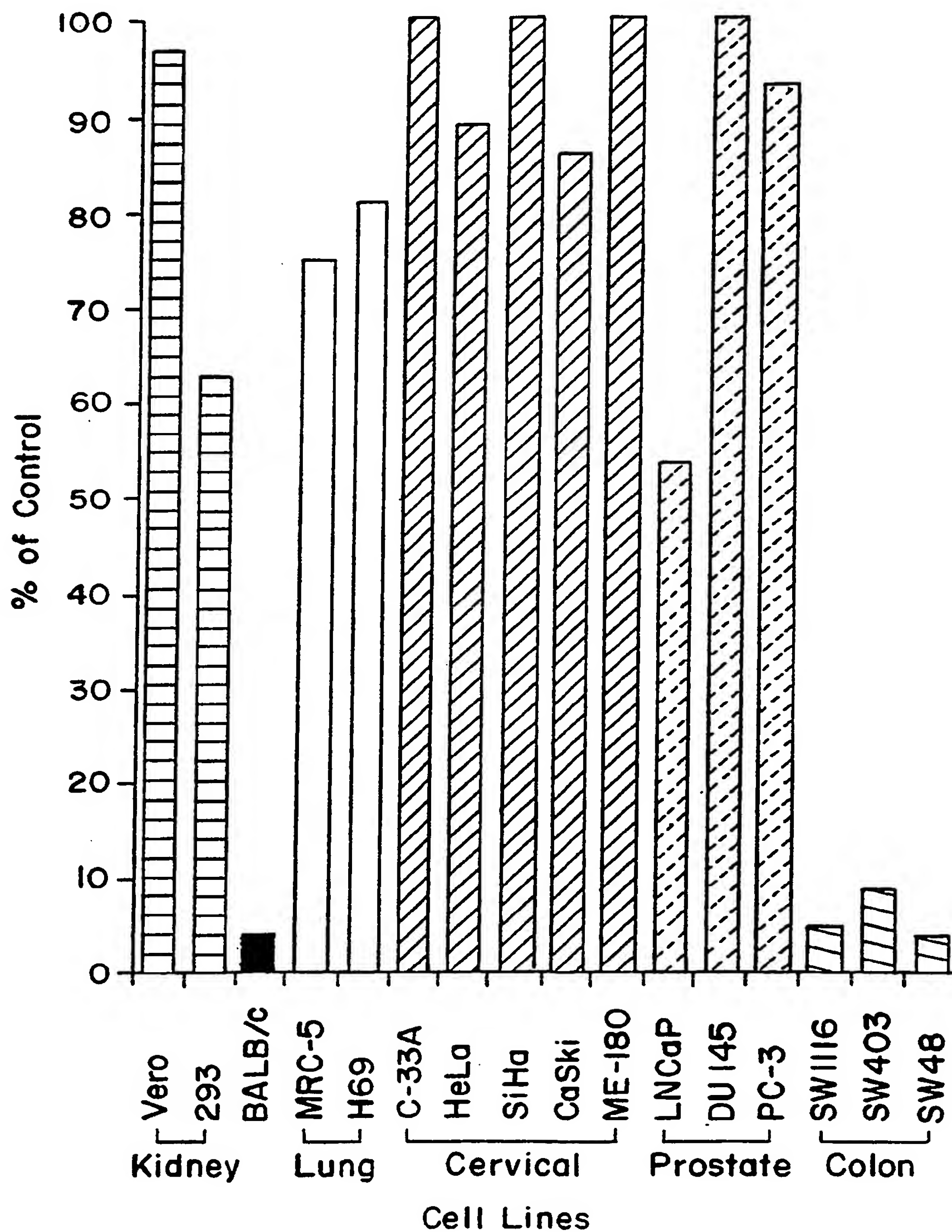
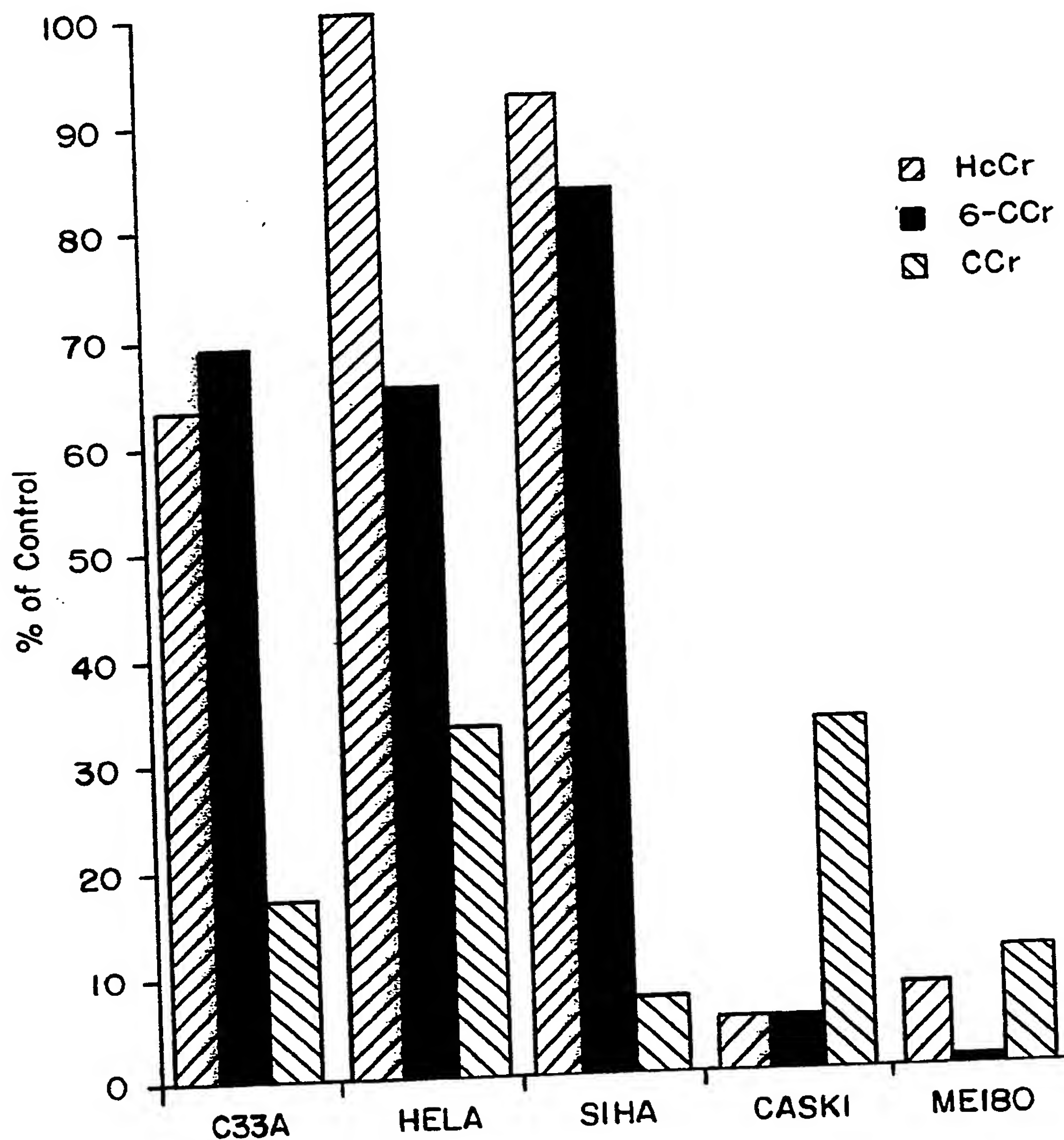


FIG. 10D  
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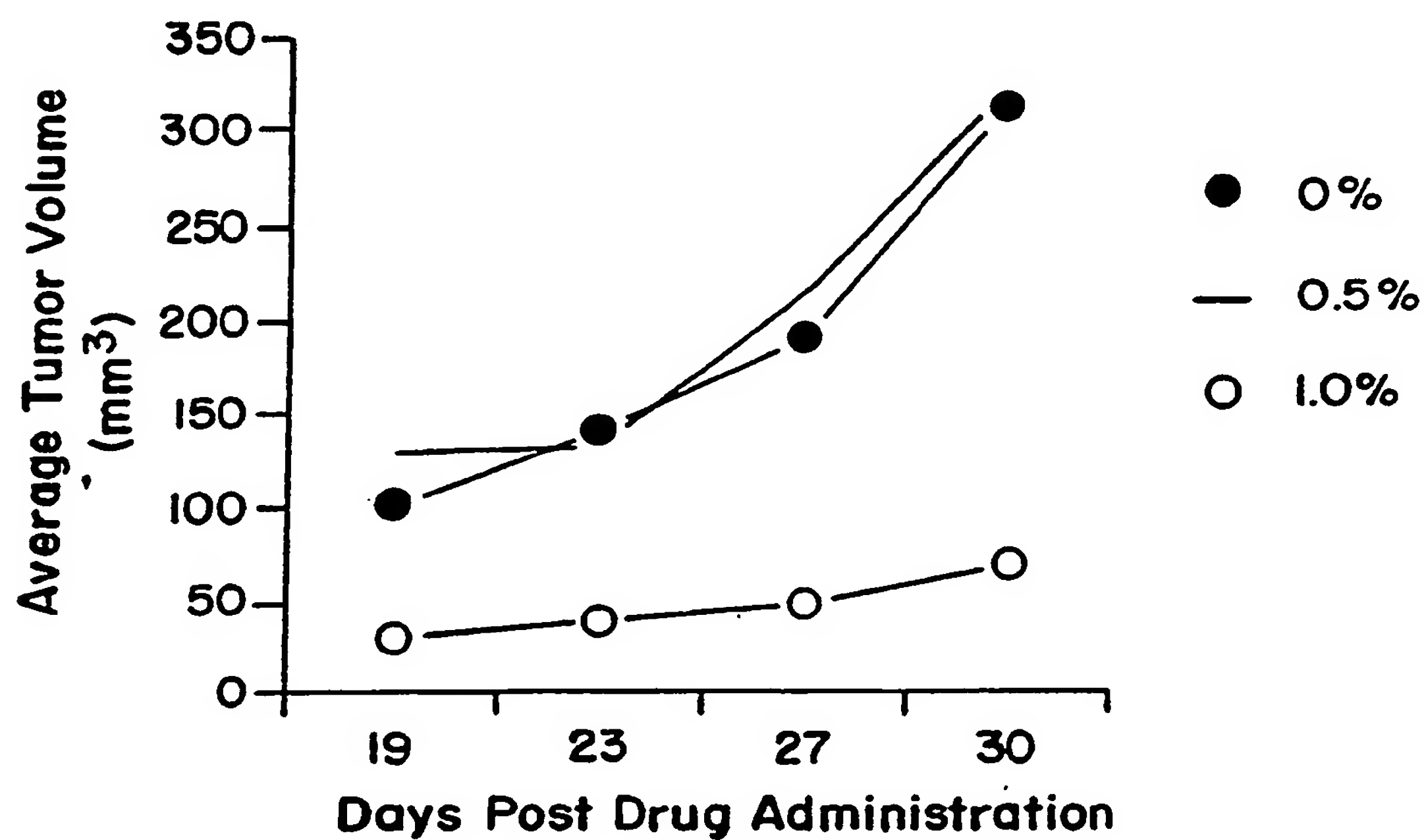


## Effects of 4mg/ml Creatine Analogs on Cervical Cell Lines

**FIG. II****SUBSTITUTE SHEET**

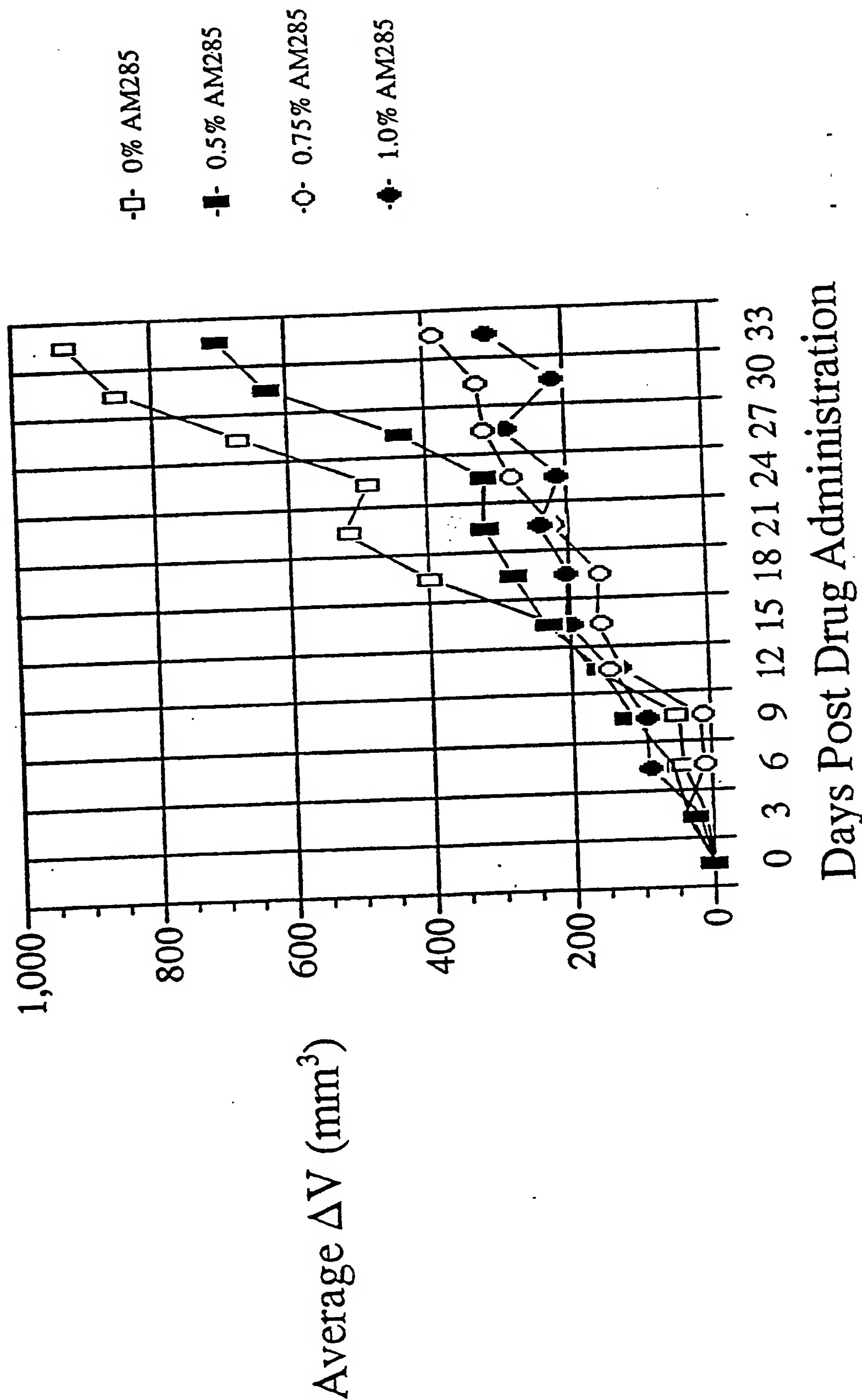


**AM285 Inhibits the Growth in Nude Mice of Transplanted Tumor Fragments Derived from the Human Colon Adenocarcinoma Cell Line SW48**



**FIG. 12**

FIG. 13  
AM285 Inhibits Growth of Established and Non-Established  
Tumors Derived from ME180 Cells in Nude Mice



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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>A61K 31/415, 31/495, 31/195</b> <b>C12Q 1/50</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 92/08456</b> <b>(43) International Publication Date:</b> 29 May 1992 (29.05.92)
<b>(21) International Application Number:</b> PCT/US91/08275 <b>(22) International Filing Date:</b> 7 November 1991 (07.11.91) <b>(30) Priority data:</b> 610,418 7 November 1990 (07.11.90) US <b>(71) Applicant:</b> AMIRA, INC. [US/US]; 373 Plantation Street, Suite 207, Worcester, MA 01605 (US). <b>(72) Inventors:</b> KADDURAH-DAOUK, Rima ; 210 Belmont Street, No. 10, Watertown, MA 02172 (US). LILLIE, James, W. ; 15 Calvin Street, Somerville, MA 02143 (US). <b>(74) Agents:</b> GRANAHAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02139 (US).		<b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report</i> <b>(88) Date of publication of the international search report:</b> 14 October 1993 (14.10.93)
<b>(54) Title:</b> METHOD OF INHIBITING TRANSFORMATION, GROWTH AND METASTASIS OF CELLS IN WHICH PURINE METABOLIC ENZYME ACTIVITY IS ELEVATED  <b>(57) Abstract</b>  A method of inhibiting growth, transformation, and/or metastasis of mammalian cells, particularly epithelial cells, in which activity of at least one enzyme, which participates in purine metabolism or regulation of nucleotide levels or the relative ratios of their phosphorylated states, is elevated. In particular, a method of inhibiting transformation, growth and/or metastasis of mammalian cells in which a DNA tumor virus, a DNA tumor virus factor or other factor which has an equivalent effect on cells has acted.		

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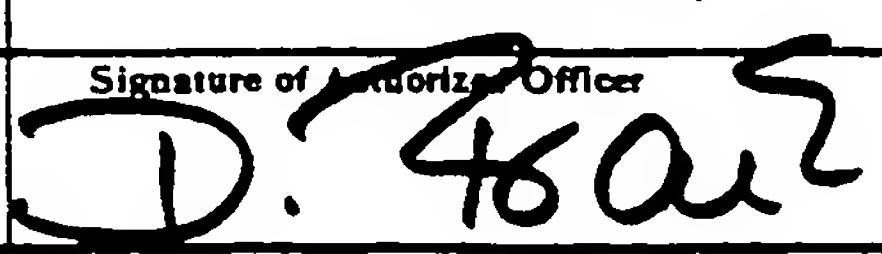
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ES	Spain				

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 91/08275

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl.5                      A 61 K    31/415                      A 61 K    31/495                      A 61 K    31/195 C 12 Q    1/50		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl.5	A 61 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>o</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	WO,A,9009192 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 23 August 1990, see the abstract; page 46, line 8 - page 47, line 7; page 49, lines 1-3; claims -----	1-26
<div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p><sup>o</sup> Special categories of cited documents : <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
31-01-1992	23.06.92	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE		

Form PCT/ISA/210 (second sheet) (January 1985)

Mme Dagmar FRANK

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_ because they relate to subject matter not required to be searched by this Authority, namely: \_\_\_\_\_
2. ☐ Claim numbers \_\_\_\_\_ because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically: \_\_\_\_\_
3. ☐ Claim numbers \_\_\_\_\_ because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

FOR FURTHER INFORMATION PLEASE SEE FORM PCT/ISA/206 SENT TO YOU 04-03-1992.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims: \_\_\_\_\_
3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:   
1-26 (partially)
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.





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